The role of RASSF1A in the regulation of NOD2/RIPK2 Pathogen Recognition Pathway

By

Ahmed Samir Said

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Department of Biochemistry

University of Alberta

© Ahmed Samir Said, 2018

Abstract

Inflammatory bowel disease (IBD) is a chronic relapsing and remitting disease of the gastrointestinal tract. There are several risk factors involved in IBD pathogenesis which are environmental, microbial and genetic factors. More than 200 susceptible genes are linked to IBD and many are involved in the innate immune response towards intestinal microbes. Three of these susceptible genes are: 1) nucleotide-binding oligomerization domain-containing protein 2 (NOD2); 2) Autophagy Related 16-Like 1 (ATG16L1) and 3) Ras association domain family one isoform A (RASSF1A). RASSF1A is a tumor suppressor protein epigenetically silenced in colorectal cancer and diseases with robust inflammation such as IBD. In a recently published manuscript, we have demonstrated the importance of RASSF1A in modulating the activation of the pathogen receptor, TLR, such that it interferes with NFκB-directed inflammation. Utilizing the dextran sodium sulfate (DSS) model of acute colitis, we demonstrated enhanced inflammation and poor recovery from DSS-induced inflammation injury in DSS-treated *Rassf1a*$^{-/-}$ mice. These mice had most of the symptoms of human ulcerative colitis. Thus, the *Rassf1a*$^{-/-}$ genetic knockout model will aid in understanding the molecular drivers of colitis and in the design of new therapeutics to treat IBD. **Our hypothesis is that RASSF1A can also restrict the NOD2/RIPK2 pathogen recognition pathway by restricting how RIPK2 and NOD2 associate thus interfering with NFκB and autophagic activation.** Using *Rassf1a*$^{-/-}$ single and the *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ double knockout mice we observed that NOD2 is responsible of inducing inflammation in these mice. RASSF1A physical associates with NOD2 and regulates RIPK2 and ATG16L1, which are the downstream signalling pathway for NOD2. The use of autophagy inhibitors and RIPK2 inhibitor-1 effectively induced recovery from DSS-induced inflammation injury in both the *Rassf1a*$^{-/-}$ mice and in the *il10*$^{-/-}$ mice. RIPK2 may be novel therapeutic approaches to enhance recovery from

injury caused by induced inflammation and they would be useful in treating and protecting IBD patients from increased risk of cancer later in life.

Preface

Two previous members of Dr. Baksh lab who are Mohammed El-Kalla and Yahya Fiteih generated the preliminary data for this thesis. Mohammed El-Kalla started to investigate the inflammation outcome of both *Nod2*$^{-/-}$ and *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ mice upon DSS administration in his thesis by the title of [Biological role of the tumor suppressor protein, RASSF1A in Inflammation and Cancer]. Yahya Fiteih investigated the outcome of using autophagy inhibitor 3-MA on *Rassf1a*$^{-/-}$ mice upon DSS administration and the role of RASSF1A in regulating NOD2 downstream signaling pathway in his thesis by the title of [Drug Discovery for Inflammatory Bowel Disease]. I have continued both of their work to confirm their hypothesis under the recommendation of Dr. Shairaz Baksh. In each chapter of the results, the contribution of Mohammed El-Kalla, Yahya Fiteih and myself will be clearly stated.

Dedication

To the soul of my father, Mr. Samir Ismail Said and my beloved mother, Mrs. Sonia Sadek Elkordi.

Acknowledgements

- First, the faculty of medicine and dentistry and the Department of Biochemistry for their support and supervision.
- I would like to thank my father Mr. Samir Ismail Said and my mother Mrs. Sonia Sadek Elkordi for their encouragements and for making me who I am at the moment.
- I am very grateful to my graduate supervisor Dr. David Stuart and the assistant dean of the FGSR Dr. Naomi T Krogman for their support and guidance.
- I would like to give my thanks mainly to my supervisory committee members, Dr. Nicolas Touret and Dr. John Seubert for their support, supervision, and guidance.
- I would like to thank my supervisor Dr. Shairaz Baksh for his support and guidance.
- I am very thankful to all members in Dr. Baksh's lab for their help, assistance, and cooperation especially to Marilyn Gordon, Yahya Fiteih, and Natalia Volodko.

Table of Contents

List of Figures

List of Abbreviation

ATG	Autophagy-related
ATG16L1	Autophagy-related 16-like 1
CD	Crohn's Disease
CARD15	The other name of NOD2
DAB	3,3′-Diaminobenzidine
DSS	Dextran sodium sulfate
IBD	Inflammatory bowel disease
IKK	IκBα kinase
LC3	Microtubule-associated protein 1 light chain 3 alpha
NF-κB	Nuclear factor-κB
NOD2	Nucleotide-binding oligomerization domain containing 2
NOD1	Nucleotide-binding oligomerization domain containing 1
PI3K	Phosphatidylinositol-4, 5-bisphosphate 3-kinase
PCNA	Proliferating cell nuclear antigen
RASSFIA	Ras association domain family 1 isoform A
RIP2	Receptor-interacting protein 2
RIPK2	Receptor-interacting serine/threonine-protein kinase 1
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TLR	Toll-like receptor
TNBS	Trinitrobenzene sulfonic acid
TNFα	Tumour necrosis factor α
TRADD	TRAF2 associated death domain protein
TRAF	TNFα receptor-associated factor
TRAIL	TNF-related apoptosis induced ligand
UC	Ulcerative colitis
ULK1	Unc-51 like autophagy activating kinase 1
VDR	Vitamin D receptor

1 Chapter One-Introduction

1.1 Inflammatory Bowel Disease

1.1.1 Introduction:

Inflammatory bowel disease (IBD) is a group of chronic intestinal condition that occurs due to abnormal inflammation of the bowel [(1)]. Crohn's disease (CD) and ulcerative colitis (UC) are the two subtypes of IBD. Both subtypes have similar clinical symptoms but what differs them from each other is the region of insult and etiology. Crohn's disease can affect any part of the gastrointestinal tract from the mouth to the anus and its inflammation is transmural, while ulcerative colitis affects only the colon and its inflammation is limited to the mucosa. IBD is a complex disease that originates from an inappropriate interaction between genetic factors and environmental or microbial factors leading to elevated and abnormal immunological responses in the gastrointestinal tract [(2)]. CD is not medically curable, and the majority of the new diagnosis for IBD were in adolescence and early adulthood [(3)]. The cost of IBD treatment is tremendously high, and the number of newly diagnosed patients with IBD is expanding with time [(4)]. North America was recently reported to have the highest incidence rates and prevalence for both CD and UC since 1950. In 2006, a study indicated that the incidences and prevalence of IBD in Alberta are 27 per 100,000 and 468 per 100,000 [(5)]. In 2015 a review was published on IBD epidemiology stating that the prevalence of IBD in Alberta between 2015 and 2025 will increase from 660 to 790 per 100,000 [(6)]. The incidence of paediatric-onset IBD is already high in Canada, and for that, the overall prevalence of IBD is expected to be high in 2025 [(7)]. IBD exists in both developed and developing countries and a lot of efforts are being made to understand IBD pathogenesis that later will assist in designing the proper treatment for such disease.

Environmental, microbial, genetic and immunological factors trigger inflammatory bowel disease. These factors have a great influence on alerting the microbiome composition leading

pathogens overgrowth and increasing host susceptibility to enteric infections. This overgrowth of pathogens in a host susceptible gene will lead to dysregulation of innate immunity. NOD2, a pathogen recognition receptors, will start to encounter pathogen invasion by regulation cytokine production and inducing bacterial clearance. Upon NOD2 activation, RIPK2 gets recruited and activated by NOD2 leading to regulation of the downstream signaling pathway of NOD2 which ATG16L1 and NFκB (Figure 1).

1.1.2 Pathogenesis of Crohn's disease and ulcerative colitis:

As previously mentioned IBD results from an aberrant combination between genetic factors and environmental or microbial factors. The disturbance in the immune system and its interaction with microbes are believed to be a significant cause of IBD development. That occurs when specific environmental factors trigger such disorder in genetically susceptible hosts. The etiology of IBD remains unknown, but several studies indicated the involvement of several factors in the pathogenesis of IBD [8, 9]. In the next section, I will introduce the different factors and their involvement in IBD development.

1.1.2.1 Environmental Factors:

Several environmental risk factors play an essential role in the pathogenesis of IBD such as smoking, vitamin D deficiency, drugs, psychological element and air pollution [3]. Smoking is the most widely common environmental trigger for IBD and has a significant influence on the immune system, inflammatory pathway, gut motility and other related factors which are involved in IBD development [10]. Smoking increases the risk for CD and is associated with a higher rate of postoperative disease [11].

Vitamin D is another environmental factor for IBD and modulating the innate and adaptive immune responses are known as vitamin D [12]. Deficiency in vitamin D is associated with both

increased autoimmunity and increased IBD development [13]. Vitamin D deficiency in mice is associated with an increased susceptibility to dextran sodium sulfate-induced colitis, and 1, 25(OH) 2D3 supplementation improved the severity of intestinal inflammation [14].

The effect of aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) in the gastrointestinal tract are well recognized. Although, limited evidence is available to support the impact of aspirin and NSAIDs in triggering onset or relapse of IBD high dose, prolonged usage duration, and frequent use of NSAIDs had increased risk of worsening CD and UC [15]. Other medications involved in IBD development are antibiotics. Antibiotics are another risk medications for IBD by altering the microbiome composition in the gut. The use of antibiotic within the first year of life was among the causes of IBD development in pediatric [16].

Psychological factors were also found to be involved in the pathogenesis of CD and UC [17], individuals with low levels of stress have a reduced risk of the disease onset, and patients with depression and anxiety showed worsening in IBD [18].

Finally, air pollution was found to play a significant role in IBD development. A Recent study suggested that air pollution in developing countries may contribute to the risk of CD and UC [19]. High levels of NO2 and SO2 are found to correlate with the increased risk of both disease [20], and some contradictory studies showed that ambient air pollution in hospitals may or may not be linked to CD, and UC increased risk [21, 22]. Environmental triggers are the most consistent factors in IBD development. These factors have great influence on affecting the composition of the gut microbiome and the intestinal barrier function leading to an inappropriate response of the immune system.

1.1.2.2 Microbial Factors:

The human gut microbiome consists of approximately more than 1150 bacterial species mainly dominated by the Gram-negative, *Bacteroidetes*, and Gram-positive, *Firmicutes* [(23)]. These bacteria live symbiotically with the host and allow digestion of indigestible molecules that the human body cannot break down. They also promote the proper development of the immune system, and even they resist the colonization of pathogenic bacteria by producing antimicrobial compounds. Thus gut microbiota protects the gut epithelial barrier from the harmful effects of pathogens by preventing bacterial overgrowth and reducing host susceptibility to enteric infections [(24)]. Changes in the microbiome composition is another factor involved with high risk for IBD development [(25)]. Many studies examined CD and UC gut flora in both inflamed and non-inflamed segments and found that there is an extremely reduced biodiversity in fecal microbiome in IBD patients compared to controls [(26)]. The lack of both *Firmicutes* and *Bacteroidetes* and an over-representation of enterobacteria are characteristic of CD [(27)]. Recently, UC has been reported to have a reduction in *Clostridium difficile* and an increase in *Escherichia coli* [(27)], but CD patients were observed to have a steady rise in mucosa-associated E. coli and decrease in *Firmicutes* [(28)]. Adherent and invasive *E. coli* (AIEC) phenotype are characterized by invasion into epithelial cells and replication within macrophages were also found in CD [(29)]. These findings indicate that normal gut microbiota protects the epithelial barrier from harmful effects of pathogens by promoting immune tolerance. Alteration of the gut microbiota composition lead to immune dysregulation, and such dysregulation showed as a risk factor for IBD development.

1.1.2.3 Genetic Factors:

Over the past years, there has been a massive improvement in understanding the genetic factors involved in IBD pathogenesis [(30)]. Since the development of genome-wide association

studies (GWAS) more than 200 susceptible genes of which 135 with both CD/UC, 36 for CD-specific and 29 for UC were identified [31]. Genome-wide association study (GWAS) to identify genes involved in human disease through searching for single nucleotide polymorphisms or SNPs that occur more frequently in people with a particular disease than in people without the disease [32]. NOD2 was the first susceptible gene identified in CD through sequencing for NOD2 in a case-control study [33]. NOD2 is a pathogen recognition receptor found to interact with ATG16L1, an autophagy-related gene, upon bacterial sensing. Such interaction induces autophagy in which bacterial replication and antigen presentation are controlled [34]. Genome-wide association study showed several polymorphisms of NOD2 and polymorphism of ATG16L1 in IBD patients [35]. ATG16L1 and NOD2 polymorphisms in both epithelial cells and dendritic cells showed defective bacterial clearance and dysregulation in the immune response leading to induce inflammation [36].

With the widespread use of GWAS other single nucleotide polymorphisms (SNPs) have been identified to be associated with IBD such as IL-23 [37]. IL23R gene is a receptor for the pro-inflammatory cytokine interleukin (IL)-23 and is involved in the generation of Th17 cells. Th17 and IL-23 pathways have been linked to the pathogenesis of both UC and CD [37]. Defective IL-10, an anti-inflammatory cytokine, have also been associated with CD and UC. Studies showed that polymorphisms in the promoter region of IL-10 reduce serum levels of IL-10 and this reduction has been associated with some forms of IBD [38]. Recent progress in identifying the genetic factors involved in IBD pathogenesis holds the key to understanding what causes and maintains the inflammatory nature of IBD.

1.1.2.4 Immunological Factors:

Understanding the relationship between immune response and IBD pathogenesis has been dominated by many studies, and evidence suggested that the dysfunctions of innate and adaptive

immune response contribute to the abnormal intestinal inflammatory response in patients with IBD. Many studies in the past years have focused on the role of immune responses in the pathogenesis of IBD. These studies helped in classifying IBD into two types which are CD-driven by a T helper-1 (Th1) response, and UC pushed by T helper-2 (Th2) response [(39)]. Interferon-gamma (IFNγ) is an essential cytokine of T helper-1 that produces the proinflammatory reactions responsible for killing intracellular parasites and maintaining autoimmune responses. T helper-2 cytokines are associated with immune globulin E (IgE) promotion and eosinophilic responses. Th17, are proinflammatory cells known by their production of IL-17, have recently observed by its involvement in the gut inflammatory response in IBD [(40)]. Currently, immunological studies have focused on the innate and adaptive immune responses and their involvement in IBD pathogenesis.

1.1.2.4.1 Innate immunity:

The mucous layer that covers the intestinal epithelium is the first physical b**a**rrier that intestinal bacteria and food antigens encounter. The mucous layer of the intestine proved its importance in preventing bacterial break-through that leads to intestinal inflammation [(41)]. This barrier is regulated by secretory cells such as goblet cells and Paneth cells which are formed by the intestinal epithelium. The goblet cells main role is to secrete mucus to protect the mucous membranes, and Paneth cells synthesize and secretes antimicrobial peptides and proteins. Defective functions of these cells in IBD patients were observed [(42)]. The innate immune response is the second line of defense against pathogen invasion and its regulated by a variety of cells such as intestinal epithelial cells, neutrophils, dendritic cells, monocytes, macrophages and natural killer cells [(43)]. Innate immunity is initiated by the recognition of microbial antigens through pattern

recognition receptors including Toll-like receptors (TLRs) and NOD-like receptors [44]. GWAS revealed NOD2 mutations in CD patients, and defective NOD2 protein showed an inadequate response to a microbial constituent. NOD2 mutations represent loss-of-function leading to the reduction of both NF-κB activity [45] and antibacterial agent production by Paneth cells [46]. Other study suggested that lack of inhibition of TLR2 stimulation by NOD2 polymorphism causes activation of inflammatory pathways and excessive Th-1 responses [47]. These effects are defective in patients with NOD2 mutation 3020insC since it is a gain of function polymorphism [48]. ATG16L1 is a necessary gene in the autophagy signaling pathway and the coding mutation T300A in ATG16L1 shown with an increased risk of CD [35].

1.1.2.4.2 Adaptive immunity:

The adaptive immune system is one of the main immune system found in body. Adaptive immunity after an initial response to a specific pathogen creates an immunological memory that enhances the immune response against the same pathogen. Adaptive immunity depends on the type and number of T cells regulated. IL-12 induces Th1 cells and they produce a high amount of IFN-γ, whereas Th2 cells produce IL-4, IL-5, and IL-13 [49]. Abnormal Th1 immune response causes intestinal inflammation in CD, and both IL-2 and IFN-γ production are highly observed in CD patients when compared to UC patients or controls [50]. In UC patient's natural killer T cells found to release high amounts of the Th2 cytokine IL-13 when compared to T cells from controls or CD patients [51]. For these reasons, CD has been thought to be characterized as a Th1 immune response, while UC as a Th2-mediated disease [52]. There have been different observations about mucosal Th1 and Th2 cytokine production in IBD. Biopsies from both UC and CD patients found to release high and comparable amounts of IFN-γ when cultured in vitro [53]. IL-13 is another

cytokine that is found at a low level in the colonic mucosa of UC patients when compared to CD patients. Current studies suggested that IL-13 has an anti-inflammatory effect on mice treated with DSS [(54)] and IL-13 levels were lower than IFN-γ in intestinal biopsy samples [(55)]. Th17 cells are a subset of pro-inflammatory T helper cells defined by their production of interleukin 17 (IL-17), and they are related to T regulatory cells. The involvement of Th17 cells and their cytokine IL17A in intestinal inflammation has been extensively studied. IL-17A transcriptional level in both CD and UC mucosa in comparison to the normal gut was highly detected [(56, 57)]. Crohn's disease patients also showed abnormal B cells responses such as increased numbers of immunoglobulin-secreting cells [(58)], and serum antibodies against Saccharomyces cerevisiae antibodies (ASCA) [(59)] and neutrophils (ANCA) [(60)]. The mechanism on how B cells influence disease activity is not clear until this moment, and that is because murine models showed the suppressive role of B cells in gut inflammation [(61-63)]. Until recently, a study was done on CD's patients showing an induce activation of B cells with granulomatous tissue localization and increased molecular maturation of IgA and IgG. These regulations were blocked when CD's patients were treated with infliximab, TNFα blocker, indicating the role of chronic B cells response in patients with Crohn's disease [(64)]. Advances in immunology and genetics have clarified the importance of innate and adaptive immunity in IBD pathogenesis and understanding the interaction of different components of innate and adaptive immunity will open new knowledge in understanding the mechanism of gut inflammation. In the next section, we will discuss these different medication used to treat inflammation in IBD and their mechanism.

1.1.3 Clinical Manifestation and Treatment of IBD:

IBD is a chronic disease with symptoms ranging from being mild to severe during relapses and may decrease during remissions. The general symptoms of IBD are fever, loss of appetite, weight loss, and fatigue but symptoms related to the damage caused by inflammation of the digestive tract are diarrhea or constipation, abdominal pain, and rectal bleeding. There are several methods to diagnose IBD, but colonoscopy is mainly used to diagnose IBD. Stool and blood samples are collected in all cases of IBD for monitoring and diagnosing IBD progression. Different classes of medications treat inflammatory bowel disease, but the first step for IBD management is adjusting patient's dietary habit to reduce abdominal pain and diarrhea.

First line of treatment for IBD is the use of 5-aminosalicylates for mild to moderate cases of colitis. The mechanism of 5-aminosalicylates is blocking the production of prostaglandins and inhibiting the activation of NFκB [(65)]. 5-ASA exerts its anti-inflammatory action by acting on peroxisome proliferator-activated receptor gamma (PPARγ) in epithelial cells. PPARγ is a receptor that induces inflammatory cytokine production and thus contributes to intestinal inflammation as seen in IBD patients [(66)].

Corticosteroids are a class of anti-inflammatory compounds that usually suppress inflammation and induce relief of symptoms. They are considered as the second line of treatment to induce remission for both types of IBD (CD and UC). It acts through the downregulation of both proinflammatory cytokines such as IL-1, IL-6, and TNFα and NFκB activity by its interaction with corticosteroids receptors [(67)].

Methotrexate is a chemotherapy agent and immune suppressant. Methotrexate is the third line of treatment, and it is used when 5-ASA or corticosteroids are ineffective or partially effective [(68)]. This drug is usually used to treat rheumatoid arthritis via inhibition of purine metabolism leading to adenosine release, a potent anti-inflammatory substance. This will lead to decrease

antibody formation, inhibit cellular proliferation and decrease the production of mediators of inflammation such as interleukins and eicosanoids [(69)]. Methotrexate was found to inhibit interleukin 1-beta, a proinflammatory cytokine, binding to its cell surface receptor [(70)].

Infliximab, an anti-tumor necrosis factor alpha (TNFα) agent, has been proven to be effective to treat moderate to severe cases of IBD when the first three medications are ineffective. This drug is a synthetic antibody against TNF-α and exert their effect by binding to TNF-α receptors and preventing its activation. TNF-α is an inflammatory cytokine and one of the primary mediators of the abnormal immune response in IBD patients. Significant improvement in managing IBD was observed using TNF-α inhibitors [(71)]. The FDA approved infliximab for treating Crohn's disease [(72)], UC [(73)] and other inflammatory diseases.

All these information summarizes different medications for treating IBD. However, medical therapies not always show symptoms improvement in many patients and for that surgical intervention shown to be necessary for remission. For such reason, efforts for developing new therapeutic agents to treat inflammatory bowel disease and to minimize the final stage of IBD treatment which is surgery are still under investigations (Figure 2). Many efforts are made to understand the molecular mechanism of IBD and their pathogenesis. We will discuss in the next section the tools that have created to study the IBD pathogenesis.

1.1.4 Animal models of IBD:

Since the etiology of IBD is still unknown, several animal models have been developed for understanding the pathogenesis of IBD that will assist in discovering new therapies for IBD treatment. These animal models were developed either by using chemical induction or genetic manipulations.

1.1.4.1 Chemically induced models:

There are several chemical agents that when administrated to animals induce colitis. One of the most commonly used agents is dextran sodium sulfate (DSS). Dextran sodium sulfate is a polysaccharide that quickly dissolves in water and mice on DSS showed characteristic features resembling that of UC [(74)]. DSS is a toxic reagent to the colonic epithelial cells causing intestinal permeability and bacterial invasion [(75)]. Following DSS insult, an inflammatory reaction is triggered in the animals gastrointestinal tract, and the expression of pro-inflammatory cytokines and chemokines such as IL-1, IL-6, KC, TNF-α, and Interferon-γ are upregulated [(76)]. DSS overexpresses Toll-like receptors, and upon activation, MyD88 gets recruited leading to NFκB activation [(77)] (Figure 3). DSS effectiveness depends on several factors such as molecular weight, dosage, duration, the strain of animals, gender and microbial environment of animals [(78)]. DSS treated mice undergo histological changes such mucin depletion, epithelial degeneration and necrosis leading to the disappearance of epithelial cells [(77)]. The acute phase of DSS also shows increase apoptosis and decrease proliferation of epithelium causing relevant leaks in the epithelial barrier allowing bacterial invasion [(79)]. DSS is widely used in IBD research to study the molecular mechanism of IBD, and in this thesis, we will use the same chemical agent in RASSF1A knockout mice to investigate our hypothesis.

1.1.4.2 Genetically engineered models:

Transgenic and knockout mice have revolutionized the field of understanding IBD pathogenesis. Knockout mice have contributed to the understanding and determining the involvement of immune-related molecules in the pathogenesis of chronic intestinal inflammation. These models have established the requirements for strict regulation of the mucosal immune

response and have allowed identification of critical components involved in gut immune regulation.

RASSF1A is the first member of the RASSF (Ras association domain family) family to have tumor suppressive activity and the loss of such gene in knockout mice showed sensitivity towards DSS. *Rassf1a*$^{-/-}$ mice upon DSS administration showed induction of NF-κB activation leading to upregulation of cytokine production and reduction in survival rate. Suggesting that RASSF1A works in a pathway to restrict induced inflammation by regulating NF-κB activity through its interaction with pathogen recognition receptors such as TLR [(80)]. The mechanism of how RASSF1A regulates inflammation is the focus of this thesis.

IL-10 is one of the cytokines that establishes the inflammatory condition in the gut. Physiologically by maintaining a check on pro-inflammatory responses to normal antigens and beneficial bacteria [(81)]. Mice with Il10 gene knockout spontaneously develop chronic enterocolitis after 12 weeks of age and is characterized by massive infiltration of lymphocytes, activation of macrophages, and neutrophils [(82)]. Recombinant IL-10 administration or IL-10 supplementation proved to be a therapeutic option for colitis treatment in an Il10 knockout model [(83)]. IL-10 supplementation therapy showed to be effective in down-regulating cytokine responsible for induced inflammation such as Tumor Necrosis Factor-α (TNF-α), IL-6 and IL-8.

NOD2 [(33)] and several mutations of NOD2 gene have been identified with CD [(45)]. Two genetically engineered mouse model for NOD2 have been created to study IBD [(84)]. The first model is the NOD2 knockout model, *Nod2*$^{-/-}$ mice are born healthy, and they display no overt intestinal inflammation symptoms up to 6 months of age [(85)]. The second animal model is a knock-in mutation in the leucine-rich repeat (LRR) of NOD2 (3020insC), this was the first identified polymorphism in CD patients [(86)]. This model is characterized by a gain of function leading to

NOD2 induce activation causing an increase of cytokine production upon MDP sensing in epithelial cells [87]. Although *Nod2*$^{-/-}$ and NOD2 3020insC mice models did not develop spontaneous intestinal inflammation under germ-free environment, NOD2 3020insC mice displayed symptoms of colitis upon DSS administration in a non-germ free environment when compared to *Nod2*$^{-/-}$ mice that showed resistant to induce colitis produced by DSS [85]. These insights suggest that dysregulation of NOD2 alone is not fully sufficient to induce intestinal inflammation but other factors to IBD development in patients carrying NOD2 mutations are contributed. Genetically manipulated NOD2 and NOD2 knockout mice strains have provided mechanistic understanding on the role of NOD2 in IBD development and how NOD2 regulates inflammation is mentioned in the third section of the introduction.

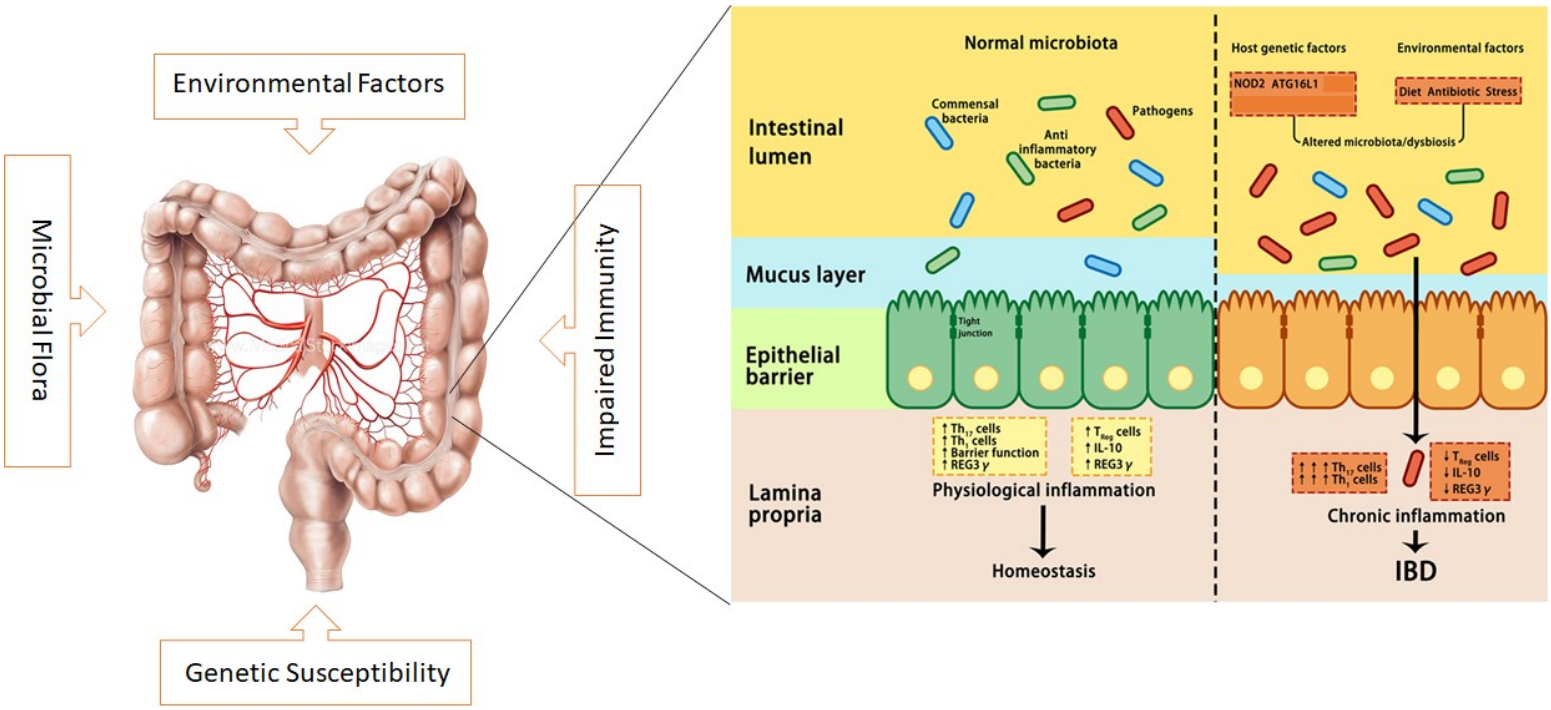


Figure 1: IBD overview: There are several factors involved in BD pathogenesis which are environmental factors, microbial flora, genetic susceptibility and impaired immunity. The Interaction between these factors contributes to the pathogenesis of IBD. Under healthy conditions, pathogens are suppressed by beneficial commensal bacteria through the induction of antimicrobial proteins thus maintaining homeostasis. In IBD, a combination of genetic factors and environmental factors lead to dysbiosis, and that will affect the barrier integrity, innate, and adaptive immunity, resulting in uncontrolled chronic inflammation [(88)].

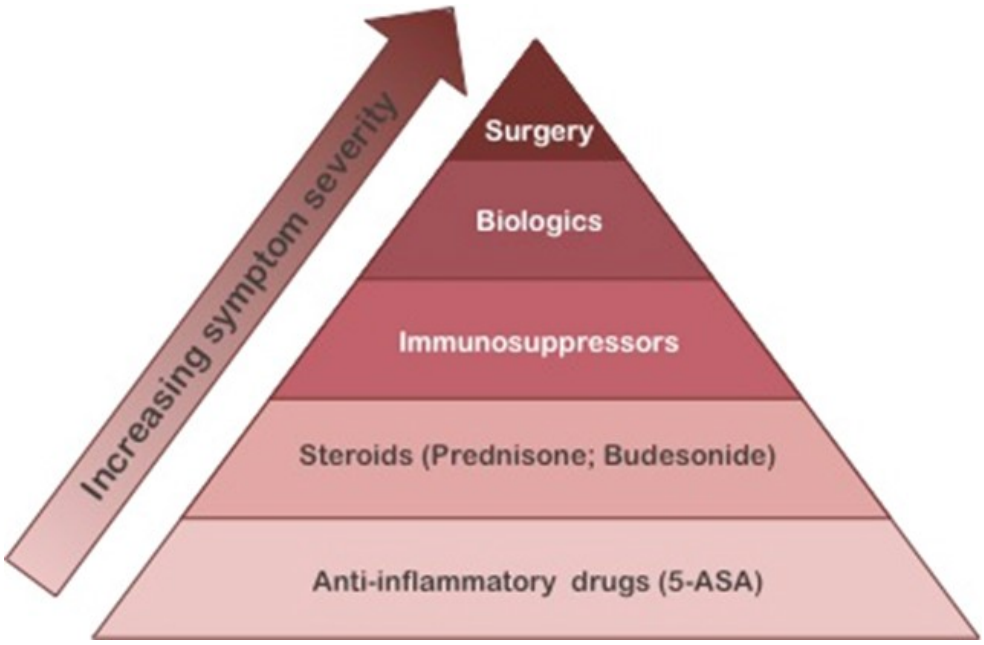


Figure 2: The medical management of IBD: Medical management of IBD starts with non-steroid anti-inflammatory drugs for the induction of remission. The second step is corticosteroids for rapid relief of symptoms and a significant decrease in inflammation. Immunosuppressors are the third option for IBD management when steroids fail to maintain remission. Anti-TNFα antibodies are steroid-sparing drugs for the maintenance of remission. Finally, if the medications showed no improvement in symptoms, then surgery will be the final stage for remission [(89)].

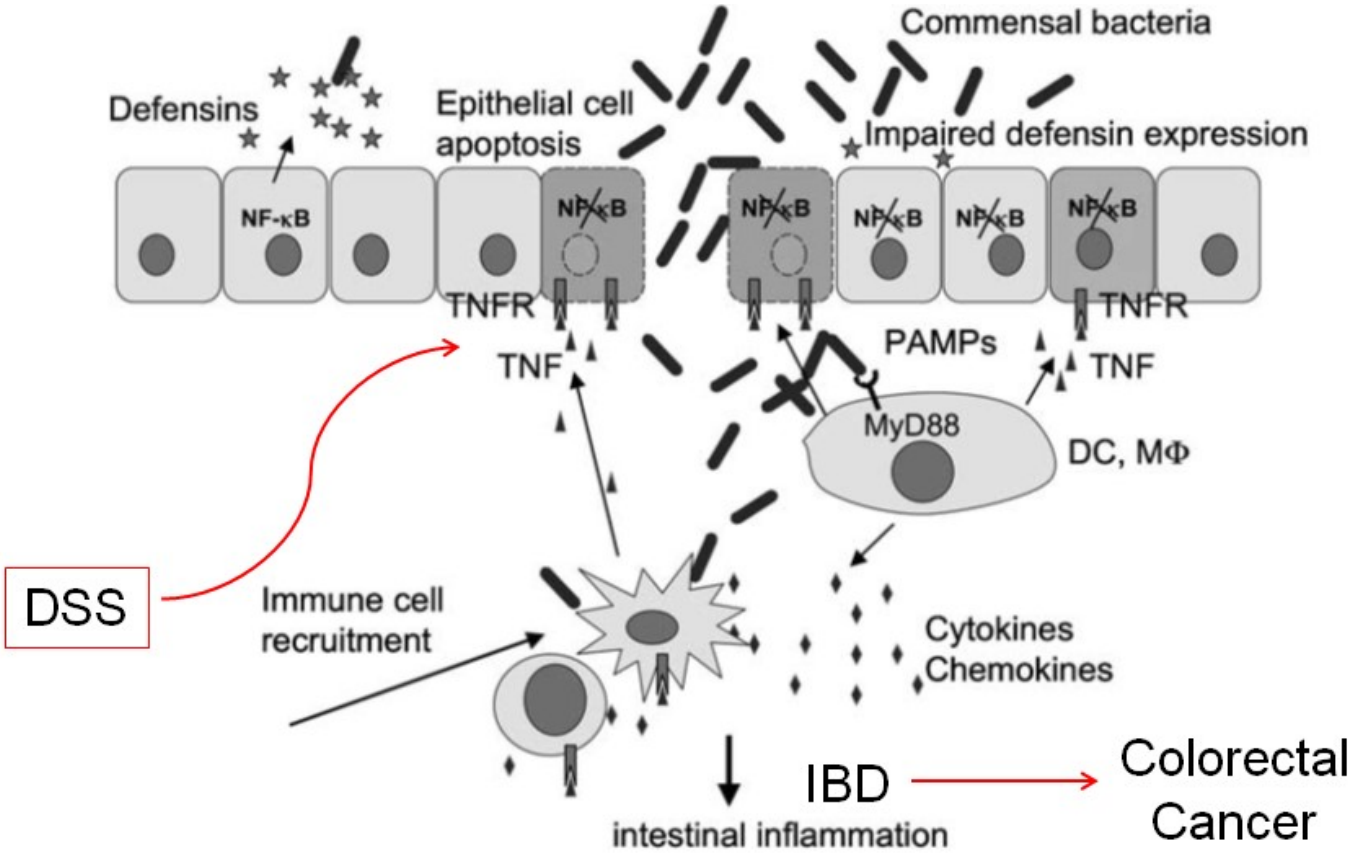


Figure 3: Dextran Sulfate Sodium model: DSS is toxic to the epithelial cells causing erosions in which increases colonic epithelial permeability. Microorganism in the intestine starts to infiltrate the epithelial cells causing activation of pathogen recognition receptors, and that will induce the cytokines and chemokines production in which will induce inflammation. In an uncontrolled system induced inflammation will later develop into colorectal cancer [(90)].

1.2 Ras association domain family member 1A (RASSF1A or 1A)

1.2.1 Introduction:

The Ras association domain family consists of 10 members with RA domain either within the N-terminal (RASSF7-10) or the C-terminal region (RASSF1-6)[91]. Although the ten members of the family are different in structure, the most common pattern between them is Ras association (RA) domain [92]. RASSF is a family of proteins that are GTP-regulated, and they are involved in transmitting signaling pathways within the cells such as proliferation, migration, cell death and differentiation [92].

RASSF1A was the first member of the RASSF family with a tumor suppression activity. RASSF1 gene has eight exons that undergo alternative splicing giving rise to 8 different isoforms (RASSF1A-RASSF1H) on chromosome 3p21.3. RASSF1C is an isoform of the RASSF1 family and is emerging as an oncogene [93]. In addition to the Ras association domain (RA domain) within RASSF1A, SARAH (Salvador-Rassf1A-hippo) domain is another domain found in the C-terminus of RASSF1A and is known for its association with pro-apoptotic kinases MST1 and MST2 [94]. ATM (ataxia telangiectasia mutant) kinase domain is found from residues 125-138 and correspond to DNA damage [95]. Finally, in the N -terminal of RASSF1A is the cytosine-rich domain (C1 zinc finger domain), and such area is characterized and demonstrated to be essential for death receptors association (Figure 4) [96]. The tumor suppression property of RASSF1A comes from its involvement in different cell biological functions. What are the different cell biological function RASSF1A involved in will be discussed in the next section.

1.2.2 The biological functions of RASSF1A

1.2.2.1 Microtubule association:

The co-localization of RASSF1A with microtubules by deletion analysis in both the N-and the C- terminus of RASSF1A revealed the importance of its interaction with microtubule [(97)]. RASSF1A was found to interact with microtubules through its interaction with MAP1B (microtubule-associated protein 1B) and MAP1S (microtubule-associated protein 1S), which are microtubule-associated proteins [(98)]. The loss of RASSF1A microtubule localization leads to inhibition of tumor suppressor properties, inhibition of death receptor-dependent cell death and the loss of tubulin stability [(98)]. *In vitro* studies on RASSF1A lacking ATM domain revealed the lack of association of RASSF1A with tubulin leading to reduction cell-cycle arrest [(99)]. Inactivation of RASSF1A in Hela cells by siRNA leads to loss of cell to cell adhesion and the development of a fibroblast-like morphology. This indicates the importance of RASSF1A association with the microtubular network to control cell migration [(100)]. MAP1S was found to interact with LC3, one of the major protein involved in the initiation of autophagy signaling. LC3/MAP1S interaction is essential for the formation of autophagosome. RASSF1A association with MAP1S proved to enhance autophagy by suppressing genomic instability and tumorigenesis [(101)]. These studies indicate that RASSF1A microtubule stabilization may have a role in autophagy regulation and that loss of RASSF1A may lead to uncontrolled autophagic response [(101)].

1.2.2.2 Control of cell cycle and mitotic progression:

RASSF1A plays an essential role in regulating the cell cycle. Several studies determined that overexpression of RASSF1A lead to an accumulation of cells in G1 phase followed by a decrease in cyclin D1 levels. Cyclin D1 is a protein required for G1 phase progression in the cell cycle. In the G1 stage, Cyclin D1 is synthesized rapidly and accumulates in the nucleus, and as the cell enters the S phase it gets degraded [(102)]. Studies have shown that RASSF1A interaction with

RABP1 (RASSF1A binding protein 1/C19ORF5/MAP1S) leads to its recruitment to the spindle poles in pro-metaphase where it can interact with Cdc20 [(103)]. RASSF1A interaction with Cdc-20 lead to inhibition of anaphase-promoting complex (APC), accumulation of mitotic cyclins A and B causing mitotic arrest [(104)]. Upon phosphorylation of RASSSF1A by Aurora A kinase, the interaction between RASSF1A and Cdc20 will not be achieved reveling APC inhibition that will lead to cyclins degradation and mitotic progression [(105)]. These observations prove the ability of RASSF1A to block cell cycle through controlling the mitotic progression and by blocking G1/S phase [(106, 107)].

1.2.2.3 Apoptosis:

Apoptosis is a programmed cell death that is initiated by intrinsic and extrinsic factors. DNA damage, unbalanced proliferative stimuli, and nutrient or energy depletion are activators of intrinsic apoptosis pathway. The intrinsic pathway involves cellular constituent degradation by a group of cysteine proteases called caspases. This path is characterized by mitochondria permeabilization and cytochrome c release into the cytoplasm. Cytochrome c forms the apoptosome, which will initiate the activation of the caspase cascade through caspase 9. Extrinsic factor includes specific death receptors such as tumor necrosis factor α (TNFα) receptor, TNFα apoptosis-inducing related ligand (TRAIL), or Fas stimulation promotes apoptosis by extrinsic pathway [(108, 109)]. Mitochondria play an essential role in regulating apoptosis by promoting the release of small apoptogenic molecules such as cytochrome c [(110)]. RASSF1A is one of the primary effector regulating the extrinsic cell-death pathway [(111)]. RASSF1A interacts with the mammalian sterile -20 like kinase 1 (MST1) and 2 (MST2) members of the Hippo pathway to modulate their kinase activity and induce cell death. MST1 and MST2 are serine-threonine kinases that are activated by auto-phosphorylation in response to stimuli such as heat shock, oxidative stress, serum

starvation and UV irradiation [112]. Both MST1 and MST2 can directly interact with RASSF1A by SARAH domain [113]. Recently, RASSF1A could modulate the activation of the MST2 pathway by controlling p73 transcriptional activity [114, 115] and also it can associate with the pro-apoptotic kinase, MST1, and function with K-Ras to promote apoptosis [116].

RASSF1A can also induce cell death by interacting with a modulator of apoptosis 1 (MOAP-1). Upon stimulation of death receptor TNF-α receptors, MOAP-1 gets recruited by TNF-α receptors to form a complex, and this allows MOAP-1 to interact with RASSF1A creating a trimeric complex leading to MOAP-1 and RASSF1A localization to the active death receptor. The association of RASSF1A with MOAP-1 lead to conformational changes exposing MOAP-1 BH3 domain to facilitate MOAP-1 binding with the BAX [96, 117]. MOAP-1 will induce conformational changes in BAX allowing it to insert itself into the mitochondrial membrane to activate cell death. BAX activation through death receptor is inhibited by the absences of RASSF1A [117], suggesting that RASSF1A is an essential mediator of extrinsic apoptotic pathways and highlights RASSF1A tumor suppression role. Although RASSF1A is a tumor suppressor, RASSF1A role in regulating inflammation in mice model of colitis was also observed. How

1.2.3 RASSF1A and intestinal inflammation:

RASSF1A is epigenetically silenced 37 types of human cancers by hypermethylation in the promoter region [118, 119]. RASSF1A was also found to be methylated in 20-52% of colorectal cancer [120], and also it was found silenced in IBD [121] and pancreatitis patients, a pre-condition diseases to cancer [122]. *Rassf1a*$^{-/-}$ mice are viable, fertile and retain expression of isoform 1C and other RASSF gene family members. These genotypes have an increased tumor incidence by 12–16 months of age especially in the breast, lung, gastrointestinal (GI) and immune system and they

develop tumors in response to chemical carcinogens suggesting that Rassf1a gene is a tumor suppressor gene [(123)].

In a recently submitted publication by our lab, mice lacking RASSF1A gene were susceptible to DSS-induced inflammation injury mimicking human ulcerative colitis [(80)]. RASSF1A knockout mice displayed clinical symptoms of murine colitis upon DSS administration. These symptoms include increased intestinal permeability, enhanced cytokine/chemokine production, elevated NF-κB activity, increased colonic cell death and epithelial cell injury [(80)]. *Rassf1a*$^{-/-}$ mice older than six months also showed enhanced susceptibility to spontaneous inflammation of the gastrointestinal tract (unpublished observations).

These data suggested that RASSF1A restricts NF-κB activation upon DSS administration by interfering with membrane complex (TLR4-MYD88/Traf6/Irak2/4) formation [(80)]. The restriction of NF-κB activity leads to the reduction in tyrosine phosphorylation of Yes-associated protein (YAP), a transcriptional co-activator and regulator of cell proliferation and apoptosis [(80)]. Previous studies showed that c-Abl, a proto-oncogene that is involved in the different cell process, can induce tyrosine phosphorylation of 357 YAP in response to DNA damage the same observation occurred during intestinal inflammation injury stimulated by DSS [(124-126)]. DNA damage will drive the formation of pY-YAP/p73 complex to activate pro-apoptotic gene expression, especially Bax [(127)]. Enhanced Y357 YAP phosphorylation in *Rassf1a*$^{-/-}$ knockout mice lead to induce in Bax expression and increased cell death [(80)] (Figure 5). These data suggest the critical role of RASSF1A in regulating inflammation in *Rassf1a*$^{-/-}$ knockout mice from DSS induced inflammation, and the loss of RASSF1A showed poor survival and recovery under the insult of DSS. *Rassf1a*$^{-/-}$ mice proved to be a compatible model in understanding the molecular mechanism of inflammatory bowel disease under the influence of DSS.

ITCH is an ubiquitin-conjugating enzyme that has a negative regulatory effect on NOD2 activation. When NOD2 gets activated by sensing both Gram-positive and Gram-negative bacteria, it recruits RIPK2. RIPK2 undergoes tyrosine autophosphorylation at Y474 and ubiquitination by inhibitor-of-apoptosis (IAP) proteins. IAPs have been demonstrated to bind and promote K63 polyubiquitination of RIP2 at lysine 209 (K209), leading to the activation of downstream signaling pathways. ITCH mediates ubiquitination of IAPs leading to IAPs lysosomal degradation and RIPK2 downregulation [(128)]. In a recent paper, ITCH was observed to degrade RASSF1A. In response to Transforming growth factor β (TGF-β), RASSF1A is recruited to TGF-β receptor I and targeted for degradation by E3 ubiquitin ligase ITCH. RASSF1A degradation will lead to the association of Hippo pathway effector YAP1 with SMADs. This complex then enters the nucleus and acts as a transcription factor for various genes [(129)]. These approaches indicate the existence of a possible correlation between RASSF1A and NOD2.

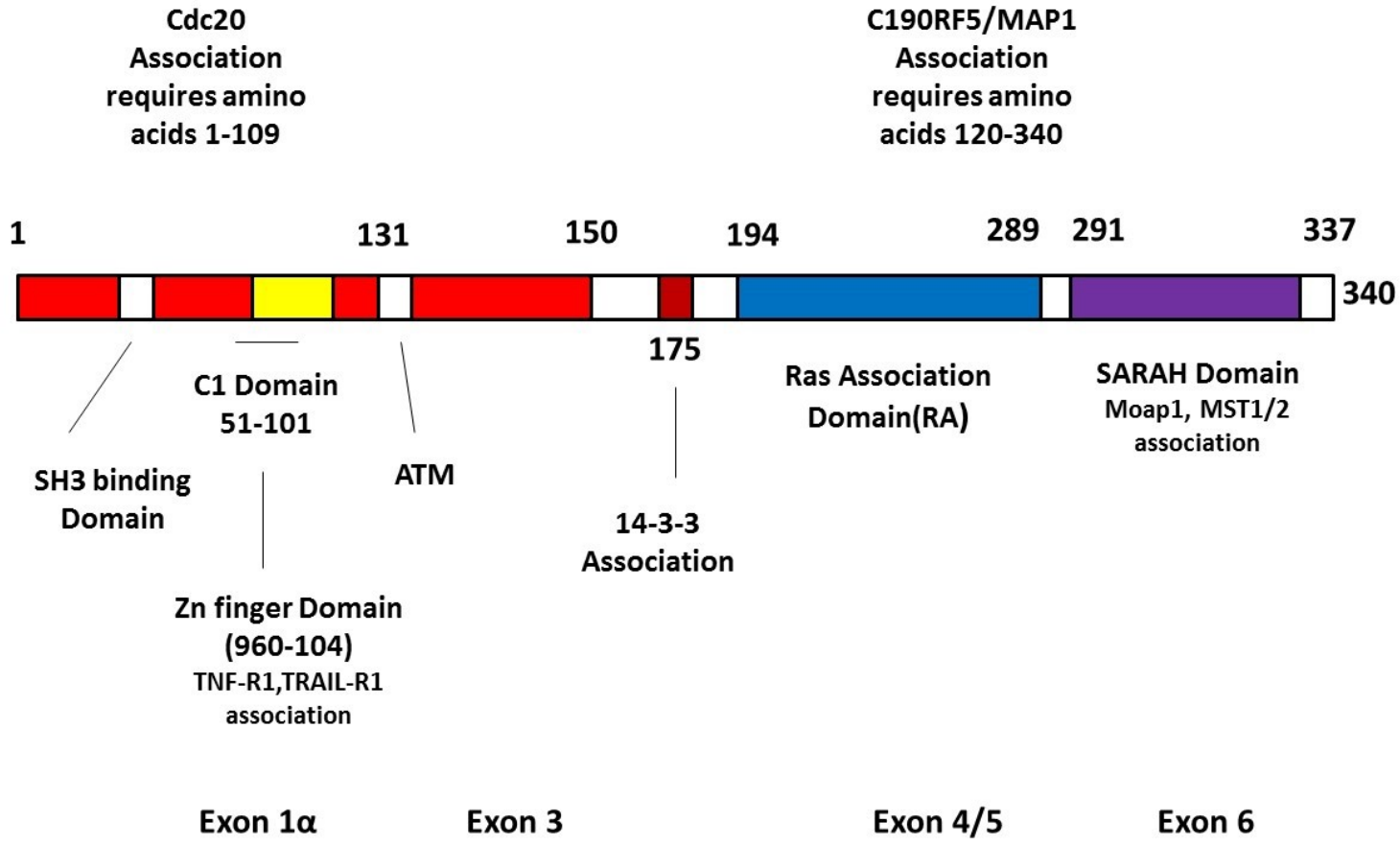


Figure 4:Schematic diagram of RASSF1A and its different functional domains; the SH3 binding domain of RASSF1A which is conserved in mouse and human forms of RASSF1A, the C1 zinc finger domain essential for TNF-R1 and TRAIL-R1 association, the ATM (Ataxia telangiectasia mutated) phosphorylation site, ATM is a serine/threonine protein kinase that is activated upon double strand breaks and apoptosis and the binding sites for several RASSF1A effector proteins are shown. The Ras association domain (RA) may potentially associate with the Ras family of oncogenes. The SARAH domain associated with the sterile-20 like kinases, MST1 and MST2. Positions of exons and amino acids are also indicated. (Adapted from El-Kalla et al.; 2010)

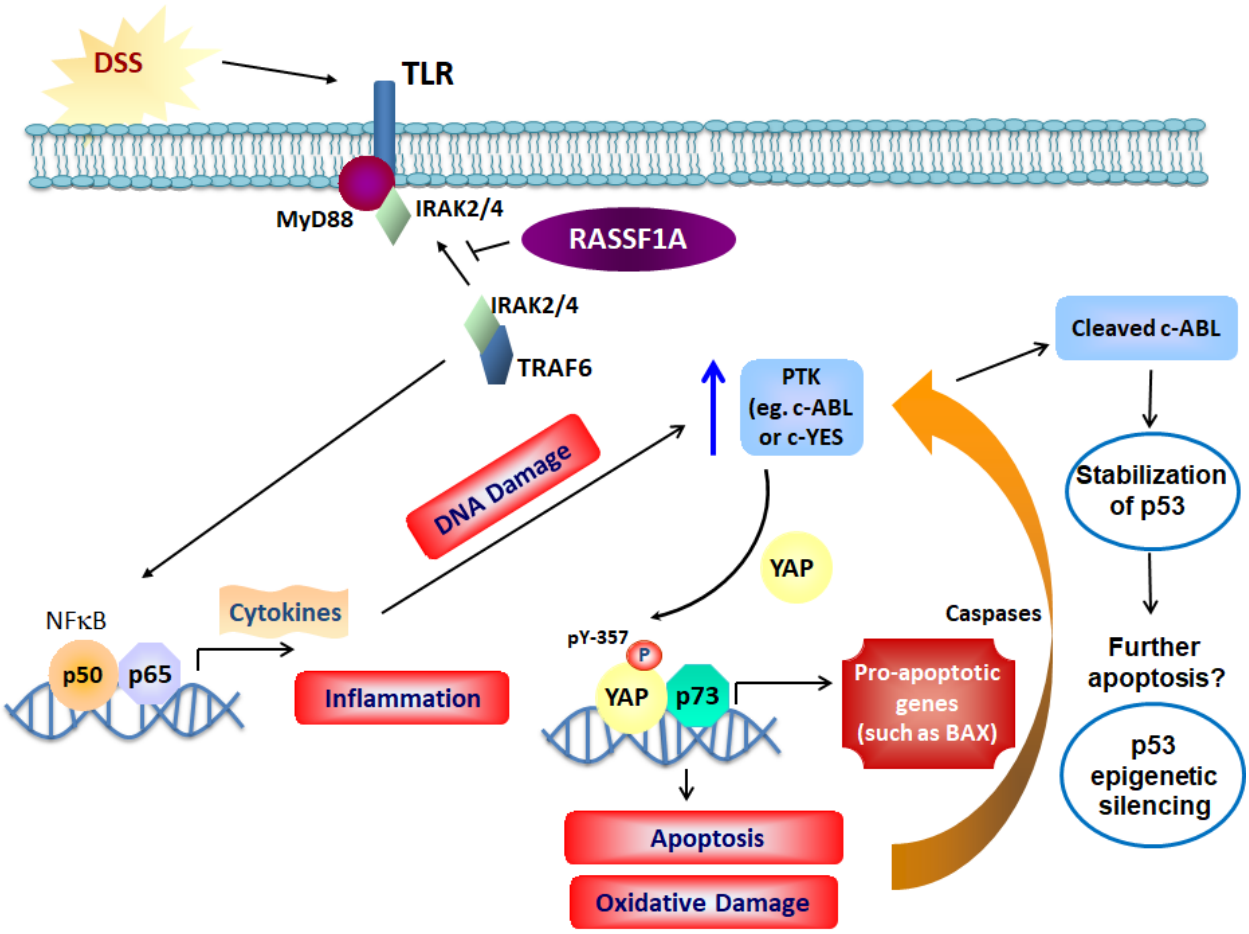


Figure 5: Model for RASSF1A regulation of NFκB and YAP: RASSF1A restricts NFκB activity by interfering with the ability of membrane-proximal TLR/MyD88/TRAF6/IRAK2/4 to promote downstream signaling to NFκB. This results in the interference of NFκB-dependent gene transcription and the activation of inflammatory pathways. Through regulating NFκB activity (and early increases in DNA damage), RASSF1A indirectly regulates the activity of a PTK to tyrosine phosphorylation YAP. Increased PTK activity would drive tyrosine phosphorylation of YAP and increased pY-YAP/p73 transcriptional up-regulation of pro-apoptotic genes such as Bax. Increased levels of Bax results in apoptosis, intestinal inflammation, oxidative (and DNA damage) and colonic injury. Sustained levels of apoptosis will result in the pro-apoptotic cleavage of c-Abl

to stabilize p53. Accumulated p53 can further promote cell death and further colonic injury and poor recovery following inflammation insults [(80)].

1.3 NOD-like receptor protein 2:

1.3.1 Introduction:

The innate immune response is a line of defense against pathogens and endogenous stress. The rapid innate immunity is regulated by a group of pathogen recognition receptors (PRRs) found on the cell membrane or in the cytoplasm [130]. NOD-like receptors (NLRs) are intracellular PRRs that responds to intracellular invading pathogens and stress signals generated within the cell [131]. There are 23 types of NLRs proteins, they all have the same C-terminal leucine-rich repeat (LRR) domain, and they all share a central nucleotide-binding domain (NACHT domain). But what makes them different from each other is their specific N-terminal domain [132]. The NLR family are classified according to their N-terminal domain into four subfamilies: NLRA, NLRB, NLRC, and NLRP. These subfamilies contain an acidic transactivation domain, baculovirus inhibitor repeat (BIR), caspase recruitment domain (CARD) or pyrin domain (PYD), respectively [133] (Figure 6). The LRR domain acts as a sensor for either pathogen-associated molecular patterns (PAMPs) such as pathogens, or danger-associated molecular patterns (DAMPs) such as endogenous stress signals. Upon peptidoglycan binding, NLRs homooligomeric and act as a signaling platform for the assembly of adaptor and effector molecules [132]. The first two members of the NLRCs to be identified are nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and nucleotide-binding oligomerization domain-containing protein 2 (NOD2).

1.3.2 NOD1 and NOD2 differences and similarities:

NOD1 (CARD4) and NOD2 (CARD15) receptors, are NLRC proteins and were the first of the NLRs to be identified [134, 135]. NOD1 is expressed ubiquitously in every cell while NOD2 is found mostly in several types of epithelial including Paneth cells which are located in the lining

of the intestine [136]. Not only the expression levels of these proteins are different but also the type of ligands they respond to are different. Both NOD1 and NOD2 receptors can sense peptidoglycan which is a significant component of the bacterial cell envelope in both Gram-positive and Gram-negative bacteria [137]. NOD1 receptors recognize γ-D-glutamyl-mesodiaminopimelic acid (iE-DAP), found in some Gram-positive bacteria and all of the Gram-negative bacteria [138]. While NOD2 receptors sense muramyl dipeptide (MDP), a conserved motif found in all Gram-positive and –negative bacteria, making NOD2 a more general and major sensor of bacterial invasion [139].

Both NOD1 and NOD2 are kept in an inactive monomeric state in the absence of pathogenic ligands by auto-inhibitory molecular interactions, whereby the LRR domain binds to its own NACHT domain, prohibit oligomerization [135]. Inactive NLRs are stabilized by forming a complex with chaperones proteins such as heat-shock proteins (HSPs), HSP70 or HSP90, avoiding pre-mature degradation of NLRs [140, 141]. These complexes are believed to contribute to bacterial tolerance by preventing excessive inflammatory response in regions where trillions of bacteria reside such as the intestine [142] (Figure 7).

Upon pathogen recognition and binding, NLRs undergoes a conformational allowing them to homoligomerise and become activated via their NACHT domains [131]. Active NOD1 and NOD2 can transduce the bacterial signal by ubiquitination/phosphorylation cascade that starts with the recruitment and activation of receptor-Interacting Serine/Threonine-Protein Kinase 2 (RIPK2) [143]. RIPK2 kinase subsequently becomes polyubiquitinated at lysine-63 (K63) a reaction catalyzed by three E3 ligases, thereby assisting the activation and binding of the transforming growth factor β-activated kinase 1 (TAK1), TAK1 binding protein 2 (TAB2) and TAK1 binding protein 3 (TAB3) complex [144]. TAK1 becomes active when associated with its complex components, and such activation will recruit the IKK complex for activation. The IKK complex

consists of an inhibitor of κB kinase α (IKKα), an inhibitor of κB kinase β (IKKβ) [145] and inhibitor of κB kinase γ (IKKγ), also known as an NF-κB essential modifier (NEMO) [146]. Activation of IKKβ will phosphorylate the NF-κB inhibitor; inhibitor of κBα (IκBα). Upon phosphorylation of IκBα, IκBα gets polyubiquitinated at K48 causing it to dissociate from the NF-κB transcription factor heterodimer (p50-p65). The released inhibitor then gets degraded by the proteasome allowing NF-κB translocation to the nucleus. In the nucleus, NF-κB binds to κB sites in the DNA and leads to the transcription of pro-inflammatory cytokines [147]. Not only TAK1 kinase activates NF-κB but also phosphorylates the mitogen-activating protein kinases (MAPKs) which are c-jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK). These kinases are the upstream mediators of the activator protein-1 (AP-1) transcription factor. AP-1 gets translocated to the nucleus where it promotes the expression of pro-inflammatory mediators [137, 148] (Figure 8).

1.3.3 NOD1/NOD2 and inflammatory bowel disease:

Since the discovery of loss- and gain-of-function polymorphisms in NOD1 and NOD2, many studies have contributed to understanding the role of these proteins in regulating chronic inflammation. mRNA expression studies revealed the involvement of NOD1 and NOD2 in a wide range of inflammatory diseases such as inflammatory bowel disease (IBD), autoinflammatory disease, rheumatoid arthritis, allergy, cardiovascular and metabolic diseases, but in this chapter, my primary focus will be on the role of NOD1 and NOD2 on inflammatory bowel disease.

UC patients revealed an increase in NOD1 mRNA expression level in colon epithelial cells when compared to biopsies taken from a patient without the disease. The expression of NOD1 originated from the same UC patients during remission was reduced when compared to control

biopsy. These observations highlight the contribution of NOD1 in UC chronic inflammatory state [(149)].

Intestinal epithelial cells are exposed to millions of bacteria. However, these cells are able to keep the intestine healthy without causing excessive inflammation [(150)]. The commensal microflora plays an important role in preventing pathogenic invasion and colonization by restoring the epithelial barrier and assisting immune cells in the mucosa to regulate basal immune response [(151)]. Both NOD2 and commensal bacteria function together in regulating the expansion of commensal communities. Commensal bacteria promote NOD2 expression, and such expression will in turn negatively feeds back and prevents overexpansion of commensal bacteria [(152)]. Indicating the importance of NOD2 in maintaining a peaceful balance between the microbiome and host immune responses. A disruption in such balance will lead to dysbiosis, whereby pathogenic bacteria begin to overwhelm symbiotic bacteria causing the destruction of the intestinal epithelial barrier allowing bacterial invasion and that will be associated with inflammation [(152, 153)]. Defective NOD2s are linked to changes in the patterns of the microbiome and epithelial barrier integrity. Knockdown of NOD2 expression significantly weaken bacterial-killing ability by Paneth cells and significantly enhanced colonization by the pathogenic bacteria *Helicobacter hepaticus* in mice [(154)]. Paneth cells are cells residing at the bottom of the intestinal crypts, and they are the critical effectors of innate mucosal defense. These cells release an antimicrobial agent α-defensin in the gut to regulate hemostasis, and the disruption of its function due to NOD2 deficiency may attribute in induce inflammation [(155)].

Most available data showed no link between NOD2 mutations and UC. Juyal et al. in 2007, showed an association between SNP5 in CARD15/NOD2 and UC in North Indians. This polymorphism leads to an amino acid change from proline to serine (P268C) in the nucleotide

binding domain (NBD). SNP5 was significantly more frequent among UC when compared with controls (17% versus 12%) [156]. Whereas In 2013 G. Walker et al., published a paper on genetic susceptibility of a different ethnicity to ulcerative colitis. In this study, he observed no significant differences in frequency in SNP5 NOD2 variant and UC patients in Indian Asians and white northern Europeans. In this study, there was no difference between Indian UC patients and controls (11% versus 15%) [157].

NOD2 deficient mice have shown altered bacterial content in their terminal ilea and an increase of both *Bacteroides* (Gm-ve bacteria) and *Firmicutes* (Gm+ve bacteria) [151, 153]. Dysbiosis, disruption in microbial balance, has been found in CD patients that have polymorphisms in both NOD2 and/or autophagy related 16 like 1 (ATG16L1) genes. However, further investigations on the true role of this bacterial imbalance showed they result from these genetic mutations [155, 158]. Since NOD2 was identified to be linked to CD [33, 86]. Extensive research on the link between NOD2 polymorphisms and CD identified three polymorphisms within or near to the LRR region. One of these polymorphisms results in the form of a frame-shift mutation (L1007fs) while the other two are missense mutations (R702W and G908R) [86, 159]. Individuals who are heterozygous for NOD2 variants have a 2-4 fold increased the risk of developing CD. Instead, individuals who are homozygous for these NOD2 polymorphisms have an additional risk by 20-40 fold [160]. Regardless of this increased risk, most individuals do not develop chronic inflammatory disorders even if they carry two NOD2 variants. Knock-In (L1007fs) NOD2 mice model raised in a specific-pathogen-free environment did not develop intestinal inflammation [161], suggesting the involvement of either microbial or environmental factors as mediators for inducing inflammation developed in patients carrying NOD2 polymorphism. These polymorphisms are a loss-of-function of NOD2 responsiveness to MDP. NOD2 maintains the intestinal epithelial barrier

by regulating antimicrobial agent release and sustaining bacterial levels and population types. The loss of function showed loss of NOD2 protective function against invading pathogens leading to increased inflammation by NOD2-independent pro-inflammatory pathways [(161, 162)]. Early studies in ileal CD patients holding the NOD2 L1007fs polymorphism in Paneth cells showed a reduction of α-defensin release in these patients [(45, 163)]. However, recent data suggested that this decline is a secondary effect of the NOD2 loss of function. A defective NOD2 will cause excessive inflammation and impairs the release of α-defensin [(164)]. The excessive inflammation of defective NOD2 is due to impaired bacterial clearance of NOD2 by ATG16L1 recruitment.

After all, ATG16L1 and NOD2 physically interact with each other and they both play a role in pro-inflammatory signaling and bacterial clearance. Several theories to explain the influence of ATG16L1 in NOD2-associated inflammation has been raised. One hypothesis proposes that ATG16L1 assists MDP trafficking within the cell and assisting NOD2 signaling [(34)]. Another study proposes that ATG16L1 is a negative regulator for NOD2 signaling pathway, it suggests that ATG16L1 dysregulate NOD1 and NOD2 pro-inflammatory signaling by blocking K63 polyubiquitin tag to the RIPK2 leading to inactivation of RIPK2 [(165)]. A third study suggested that upon bacterial invasion, NOD1/NOD2 activation and autophagy are promoted in parallel to pro-inflammatory signaling to assist bacterial degradation [(34)]. This theory was supported by research done on individuals carrying NOD2 and ATG16L1 variants and that revealed defective innate immune responses and bacterial clearance. Since NOD2 variants will not respond to invading pathogens effectively and the cell will counter pathogen invasion by enhancing the adaptive immune response via excess monocyte activation and phagocytosis. Excessive phagocytosis results in bacteria accumulation within the interior of the cell due to defective autophagic response caused by ATG16L1 polymorphism [(166)]. Current knowledge has identified

a strong link between NOD2/ATG16L1 and IBD development. These observations are still relatively in the early stage of research and further analysis is required to confirm the role of this receptor in this disease.

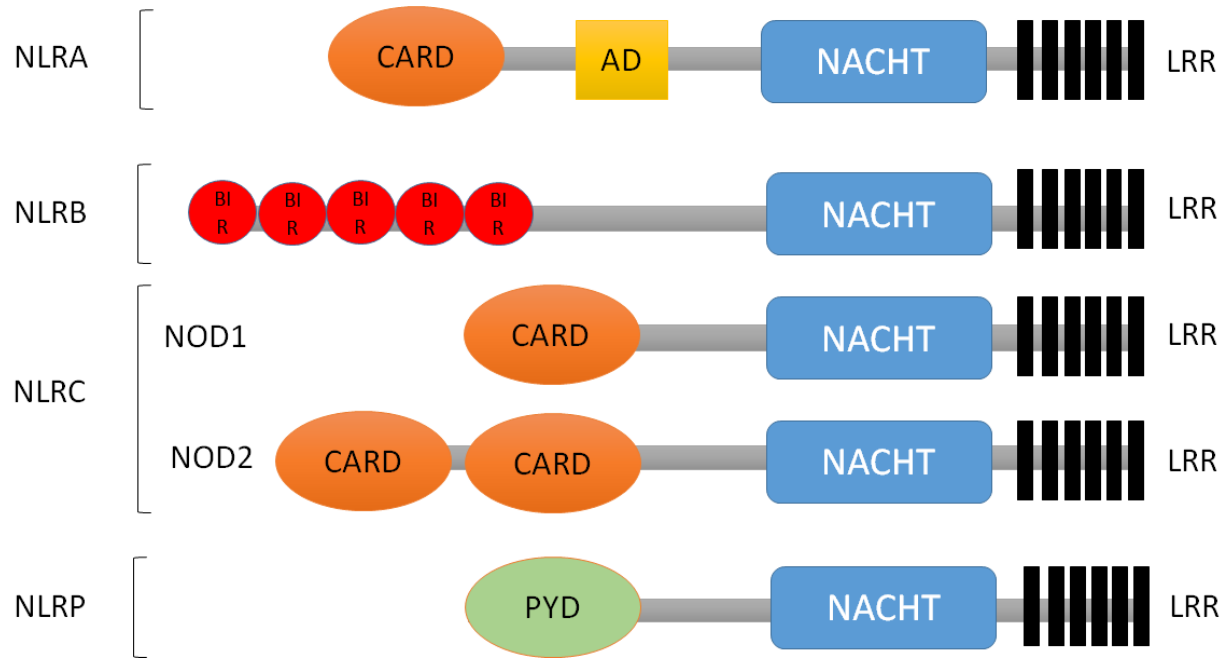


Figure 6: The NLR gene family. The NLR gene family consists of 23 members that share a tripartite structure, consisting of an N-terminal signaling domain, a central nucleotide-binding and oligomerization domain, and a C-terminal agonist sensing/ligand-binding domain. The NLR family is sub-divided into four sub-groups NLRA, NLRB, NLRC, and NLRP based on the nature of the N-terminal domain consisting respectively of an acidic transactivation domain (AD), a baculovirus IAP repeat (BIR), a caspase-recruitment and activation domain (CARD), and a Pyrin domain (PYD) [167].

.

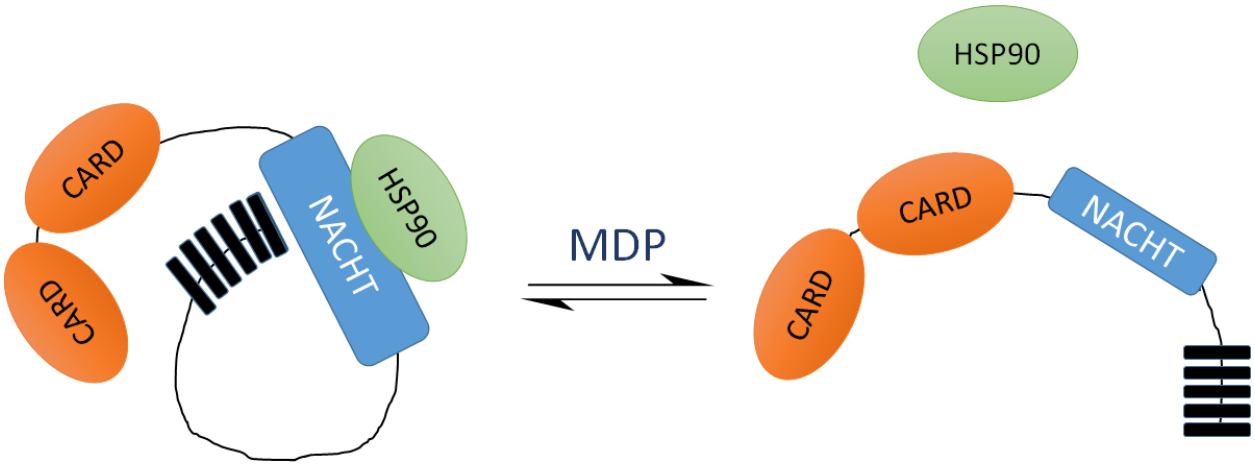


Figure 7: NLRs signaling; LRR domain binds to its own NACHT domain to inhibit oligomerization of NLRs. Inactivation NLRs are stabilized by interacting with chaperones proteins such as HSP70 or HSP90, avoiding their degradation. Upon MDP sensing NLRs undergoes conformational changes allowing them to be active through the dissociation of HSP70 or HSP90 from the complex.

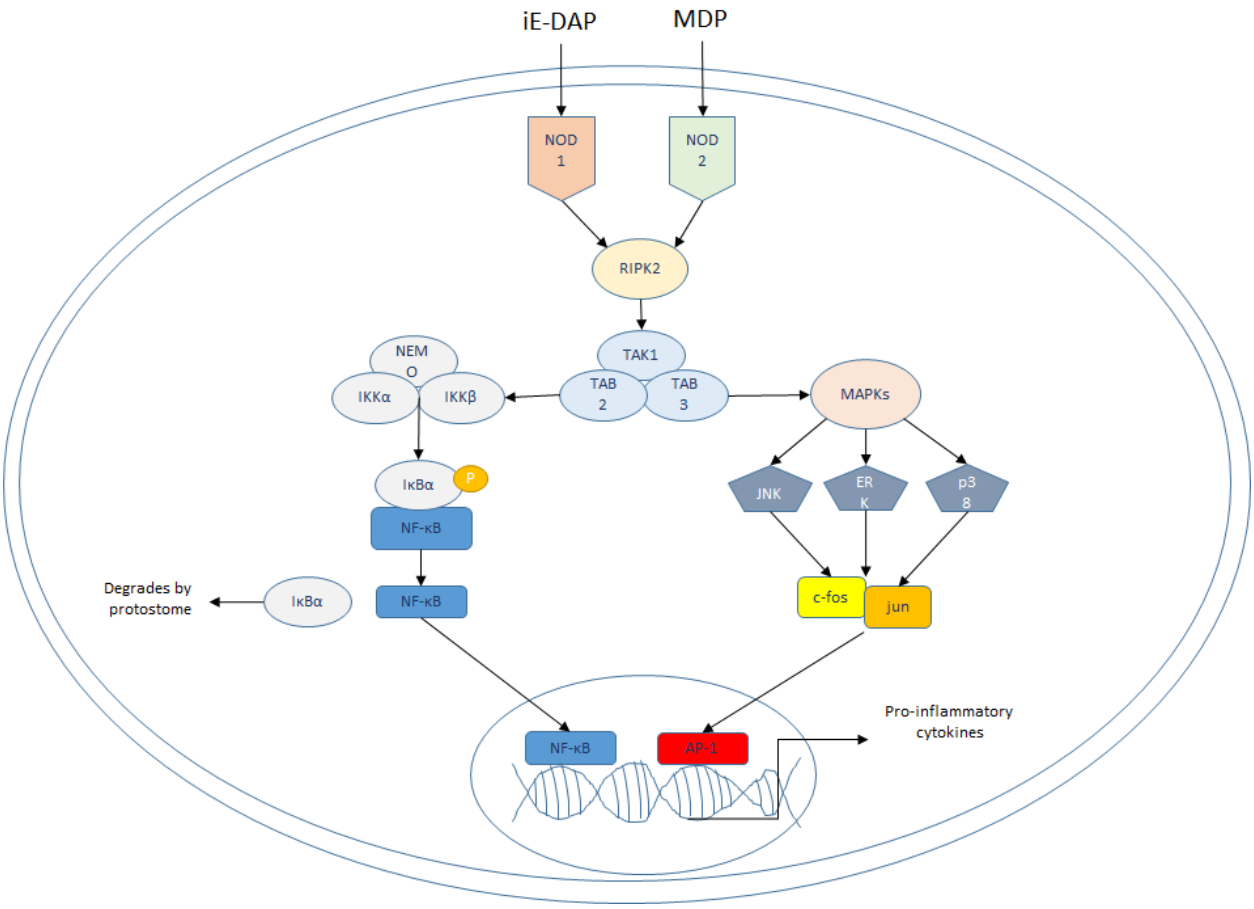


Figure 8: Model of NOD1 and NOD2 signaling cascades. NOD1 and NOD2 recognize bacterial PGNs, iE-DAP, and MDP, respectively. Following ligand sensing the NODs recruit their regular adaptor RIPK2 by CARD-CARD interactions and induce RIPK2 to undergo phosphorylation and ubiquitination. RIPK2 assists the activation and binding of TAK1, TAB2 and TAB3 complex. Active TAK1 will recruit the IKK complex for activation and activation of IKKβ will phosphorylate IκBα for degradation. NF-κB will translocate to the nucleus for pro-inflammatory cytokines production. TAK1 kinase also phosphorylates MAPKs such JNK, p38, and ERK. These kinases upon activation AP-1 transcription factor will be translocated to the nucleus to promote the expression of pro-inflammatory mediators (adapted from reference 167).

1.4 Autophagy:

1.4.1 Introduction:

Autophagy is a self-degrading system in the cell that delivers cytoplasmic constitutes to the lysosome for degradation. The cytosolic component warped for lysosomal degradation are recycled as a source of energy used by other cells [(168)]. There are three known types of autophagy which are macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is the most prevalent form of autophagy, and it is characterized by the unique formation of a double-membrane which is the autophagosome engulfing the cytoplasmic content [(169)]. The autophagosome is then fused with lysosomes and the content gets degraded [(169)]. The second type of autophagy is microautophagy; microautophagy involves engulfment of cytoplasmic cargo by inward invagination of the lysosomal membrane [(170)]. Chaperone-mediated autophagy is the third form of autophagy and it is unique for cytosolic protein degradation which is mediated by the heat shock cognate protein 70 (hsc70) and the lysosomal-associated membrane protein type 2A (LAMP-2A) [(171, 172)].

Stress, cellular starvation, damaged organelles and intracellular microbes trigger autophagy [(173)]. Autophagy is a highly controlled process mediated by protein kinases and ubiquitin-like machinery. Most of the autophagy components were identified and characterized in yeast two-hybrid, leading to the discovery of over 32 autophagy-related (ATG) genes. This highly programmed and conserved process is regulated by protein kinases and ubiquitin-like machinery into four different stages which are initiation, elongation, cargo selection, and maturation [(168)].

1.4.2 Autophagy signaling pathway:

Upon recognition of stress, starvation and bacterial invasion, ULK (uncoordinated-51-like kinases) kinase complex will initiate the formation of an isolation membrane. ULK complex activation recruits other autophagy proteins such as the class III phosphatidylinositol 3-kinase (PI3K) complex involving, Beclin-1, Vps34 (vacuolar protein sorting 34), the serine/threonine kinase p150, and Barker/mAtg14 on the isolated membrane. The activation of the PI3K complex generates phosphoinositide that will recruit other proteins to transport membrane to the growing phagophore [(174)].

Elongation of the isolated membrane occurs by two ubiquitin-like conjugation process. The first step is mediated by ATG7 and ATG10 and they will link to ATG12 to ATG5 by forming a covalent bond. ATG16L1 interacts with the ATG12 and ATG5 complex and will be localized to the phagophore. The second conjugation process is comprised of ATG4, ATG7 and ATG3, which will modify microtubule-associated protein light chain 3 (LC3) protein by attaching a phosphoethanolamine lipid and inserting the modified LC3 into the autophagosomal membrane. It has been demonstrated that the ATG12, ATG5 and ATG16L1 complex promotes LC3 lipidation and assists in the insertion of the LC3-II, the modified form of LC3-I, to the phagophore. Cargo recruitment and autophagosome closure are promoted by LC3, making it a valid biomarker in studying autophagy regulation. [(174, 175)].

Specific adaptor proteins do interact with LC3 to regulate the trafficking of protein aggregates, intracellular pathogens and damaged organelles towards the autophagosome. p62 is one of the cargo selection proteins that tags the protein aggregates, damaged organelles and intracellular pathogens with ubiquitin substrate and trafficking them to the autophagosome through binding to LC3. These substrates along with p62 will be degraded in the autophagosome by

lysosome. Showing that p62 is another biomarker for studying autophagic flux since the degradation of p62 is an indication of autophagy maturation [(174)].

The maturation of autophagosome is complete when it fuses with lysosomes to cause degradation of its contents. Both Rab7, a member of small GTPases, and lysosome-associated membrane proteins (LAMP-1 and 2) regulate the fusion of the lysosome with the autophagosome forming at the end autophagolysosomes. The cargo of the autophagosome is degraded by the action of cathepsins and lysozyme, which are lysosomal hydrolyzes, into small molecules. Degraded molecules are then transported to the cytosol for recycling or to be presented as antigens to activate the adaptive immune response [(174)] (Figure 9).

In intestinal cells, we observed under an inflammation-induced injury rodent model, the genetic loss of RASSF1A resulted in increased inflammation and colitis-like symptoms and abnormal tyrosine phosphorylation of RIPK2, the obligate kinase for NOD2. Furthermore, the increased inflammation was accompanied increased autophagy. To study autophagy regulation, p62 biomarker will be analyzed in this thesis.

1.4.3 Autophagy modulation in IBD therapy:

Many studies were adopted to investigate the outcome of using autophagy activating compounds in animal colitis models. An initial study showed that by using everolimus, a mTOR inhibitor, chronic colitis was significantly reduced in an interleukin-10 deficient mouse model by decreasing the percentage of CD4+ T cells in the colonic mucosa and reducing IFN-γ production [(176)]. Similar findings were proved in two different cases, the reports demonstrated that administrating mTOR inhibitors (everolimus and sirolimus) resulted in a marked and sustained

improvement of disease in CD patients [177]. However, everolimus showed ineffective when it was tested on CD patients with moderate-to-severe disease in a larger clinical trial [176].

Vitamin D has been identified as an autophagy regulator through modulation of calcium fluxes and the control of autophagy gene expression. It was also demonstrated that the expression of NOD2 is regulated through the activated VDR (vitamin D receptor) [178]. Linkage studies observed that polymorphisms in *VDR* (vitamin D receptor) are associated with IBD susceptibility in non-European populations in both CD and UC patients [179]. Furthermore, studies identified vitamin D deficiency as a risk factor for CD [180]. In DSS colitis model, administration of vitamin D or a VDR agonist (BXL062) resulted in improved the signs and symptoms of colitis [181], while mice fed on vitamin D deficient diet were more susceptible to DSS-induced colitis [182]. This data suggests the importance of using vitamin D as a source for autophagy regulation, as vitamin D is a natural product that has fewer side effects and may be more effective and safer for long-term treatment of chronic conditions such as IBD.

Currently, our lab is studying the effect of 3-MA (3-methyladenine), an autophagy inhibitor on a DSS colitis mouse model. 3-MA inhibits autophagy by inhibiting Phosphatidylinositol 3-kinases (PI-3K) leading to autophagosome formation blockage [183]. Unpublished data from our lab suggest that Rassf1a knockout mice have increased autophagic response after DSS treatment, which goes along with increased cell death, decreased epithelial repair and healing. Interestingly, the autophagy inhibitor, 3-MA can fully protect RASSF1A and *Il10* knockout mice from DSS-induced inflammation injury and reduces the signs of colitis. Furthermore, chloroquine partially reversed the damaging effect of inflammation- induced by DSS and increases the survival rate of *Rassf1a* knockout mice from 20% to 40%. These findings suggest the importance of using pharmacological modulators and/or pharmacological inhibitors for autophagy as a novel

therapeutic approach for IBD treatment and protecting IBD patients from predisposing to cancer later in life.

1.4.4 Autophagy and Inflammatory bowel disease:

Many genes related to autophagy have been linked to IBD development. These genes are IRGM (Immunity-related GTPase family M protein) [184], NOD2 (Nucleotide-binding oligomerization domain containing 2) [36], VDR (Vitamin D receptor) [185] , DAP (Death-associated protein) [186], autophagosome formation, ULK1 **(**Unc-51 Like Autophagy Activating Kinase 1**)** [187] and ATG16L1 (Autophagy-related 16 like1)[188] or in autophagosome maturation, LRRK2 **(**Leucine-rich repeat kinase 2) [189]. NOD2 polymorphisms were first identified in IBD patients, and later ATG16L1 polymorphism in IBD patient through GWAS was also determined. Since both NOD2 and ATG16L1 can physically interact with each for autophagic clearance of intracellular pathogens [190], many studies were implemented to understand the mechanism of these two genes polymorphism in regulating inflammation in IBD.

1.4.4.1 ATG16L1:

ATG16L1 is a major protein in the autophagy signaling pathway and has an essential role in guiding molecules of the autophagic machinery for autophagosome formation [191]. In vitro studies proved that upon knocking down ATG16L1 expression in cell lines the localization of intracellular bacteria to the autophagic vacuoles is reduced. Such observation indicates the importance of ATG16L1 in bacterial degradation through autophagy [192].

A single nucleotide polymorphism (SNP) in ATG16L1 gene has been identified to be associated with CD [193]. This SNP is common, and about 45–50% of healthy individuals carrying this risk allele showed no signs of inflammation indicating that IBD is a multi-factorial disease.

ATG16L1 is composed of several domains but the most critical domain in this gene and is essential in autophagic activity is the WD repeat domain. Threonine to alanine amino acid change at position 300 (T300A) within the C-terminal tightly conserved WD repeat domain is one of the most frequent polymorphisms associated with IBD [(194)]. Some studies proved that such polymorphism might be impaired antibacterial autophagy function, and such finding suggests that ATG16L1 (T300A) mutation contributes to IBD pathogenesis [(195)]. Other studies with healthy individuals and CD patients demonstrated that ATG16L1 expression at both protein and mRNA levels were not affected either by ATG16L1 genotype or the state of inflammation in the intestine [(35)]. Furthermore, mutations studies confirmed that this region is not essential for starvation-induced autophagy in fibroblasts [(191)]. These studies showed contradictory on the role of ATG16L1 and its polymorphism on IBD. To explore the relation between ATG16L1 and IBD, genetically manipulated mice were created for in vivo studies.

In vivo mice with functional knockout of the ATG16L1 protein was generated [(196)]. These mice die within 24 hours of birth, showing that autophagy is essential for survival in newborn mice [(196)]. Autophagy in ATG16L1 knockout macrophages was inhibited but IL-18 and IL-1β level upon treating these macrophages with Lipopolysaccharide (LPS), or other activators of the inflammasome. ATG16L1 knockout mice showed to be sensitive to DSS, and they show signs of colitis, lymphocytic infiltration and ulceration which correlates with high levels of pro-inflammatory cytokines IL-1β, IL-18 and IL-6. Interestingly, CD patients and healthy volunteers who were genotyped for ATG16L1 variant showed in hyper-production of IL-1β [(197)] suggesting the role of autophagy in regulating pro-inflammatory cytokine production.

Atg16L1 hypomorph (*Atg16L1HM*) mice were generated, and they showed 30% reduction of ATG16L1 expression level [(198)]. In-depth analysis was done on these genotypes, and it was

observed that the morphology and architecture of the intestine and the colon were not affected and also no signs of spontaneous inflammation was shown. The Paneth cells of these genotypes demonstrated transcriptional alterations, functional changes, and morphological abnormalities. Paneth cells, are secretory cells located at the base of the crypts and plays a vital role in mucosal defense through the secretion and production of anti-microbial lysozyme, peptides, and inflammatory mediators into the gut. High levels of leptin and adiponectin are transcribed from Paneth cells of *Atg16L1HM* mice, and both immunoregulators have been linked to a pathologic hallmark of CD [(199)]. Visible packaging and exocytosis of antimicrobial granules have been demonstrated in Paneth cells. Similar Paneth cell abnormalities were also detected in CD patients carrying homozygous for the ATG16L1 risk allele. Such observation indicates that this mouse model may replicate some of the pathological changes that are associated with human disease. Noting the importance of animal models in studying IBD pathogenesis and molecular mechanism [(198)]. ATG16L1 HM mice are resistant to DSS-induced inflammation indicating that reduction of ATG16L1 expression by itself is not sufficient enough for the pathologic changes found in IBD patients. Proving that IBD is a multi-factorial disease and genetic risk factor alone is not enough for disease development.

1.4.4.2 NOD2 and ATG16L1:

Studies by three different groups demonstrated ATG16L1, a protein in the autophagy signaling pathway, is triggered by NOD2 and NOD2-ATG16L1 interaction may consider susceptible factor for IBD pathogenesis. In vitro studies proved that NOD2 activates autophagy and the interaction of NOD2 with ATG16L1 were demonstrated in epithelial cells at the site of bacterial entry indicating that NOD2 is essential in guiding ATG16L1 towards pathogens entry sites on the plasma membrane to activate autophagy [(34)]. NOD2 (L1007fs) risk variant showed to

be defective in localizing to the plasma membrane unlike the wild-type NOD2 and that is because of the mutation in the sensory domain of NOD2 [(200)]. Such observation suggests that the inability of NOD2 to localize to the site of invasion might be one of the contributing factors for Cohn's disease appearance. Additionally, other studies showed that cell expressing L1007fs mutation of NOD2 are unable to target bacterial autophagosome and NOD2 and ATG16L1 localization to the plasma membrane was impaired. Indicating that NOD2 and ATG16L1 membrane localization is essential for bacterial clearance [(34)].

In 2010, Homer et al. indicated that activation of NOD2 signaling pathways regulates autophagy, MAPK and NF-κB [(36)]. NF-κB activation through NOD2 upon MDP stimulation was reduced when autophagy was blocked by inhibitors or by knocking down ATG16L1 expression [(36)]. These results suggest that autophagy is a novel mechanism that can be regulated by NOD2 during an intracellular bacterial invasion. However, this effect depends on cell type and function, as NF-κB activation by MDP stimulation in human epithelial cells and macrophages was similar to cells expressing wild-type ATG16L1, but bacterial killing enhanced by MDP in epithelial cells was impressively impaired when compared to macrophages. In 2013 ATG16L1 was revealed to be a negative regulator of NOD2 inflammatory responses and ATG16L1 knockdown showed to promote NOD2 driven cytokine production [(165)]. Indicating that ATG16L1 has an inhibitory role in NF-κB activation via NOD2 activation. Therefore, there is a contradiction between the two studies described above, suggesting more investigations have to be done to confirm the role of autophagy-related proteins specifically ATG16L1 in regulating NOD2 driven inflammatory responses. NOD2 stimulation by bacteria and activation of autophagy provides a functional link between NOD2 and ATG16L1 both of which are related to IBD. Furthermore, it highlights that

the impairment of NOD2 and autophagy signaling might influence intestinal inflammation and contributes to IBD pathogenesis.

Defective autophagy was linked to IBD pathogenesis, with evidence showing that regulating autophagy may be therapeutically beneficial. A recent paper studied the effect of IBD medications on modulating autophagy signaling pathway [(201)]. Another study in 2016 published the outcome of autophagy inhibitor 3-methyladenine (3-MA) on corticotropin-releasing hormone (Crh), knockout mice. Corticotropin-releasing hormone is a peptide hormone involved in the stress response and intestinal biopsies from patients with IBD identified induced Crh levels when compared to control samples. DSS administered *Crh*$^{-/-}$ mice treated with 3-MA showed improvement in survival and reduction in inflammation [(202)].

Gathering all of these data, we hypothesize that **RASSF1A may have a role in regulating NOD2 signaling by preventing RIPK2 from associating with NOD2 and thus interfere with NF-κB and autophagic activation.** We aim in testing our hypothesis by using the *Rassf1a*$^{-/-}$ knockout mice and exploring the different objectives that are created for supporting our hypothesis. These objectives of my thesis were; (i) if the NOD2/RIPK2 pathway is responsible for triggering inflammation in the RASSF1A knockout mice upon DSS-induced injury then the outcome in the *Rass1a*$^{-/-}$*Nod2*$^{-/-}$ mice upon DSS administration should be blunted. (ii) if RASSF1A associates with NOD2 and regulates NOD2 signaling pathway by regulating RIPK2 and ATG16L1. This can be answered by immunoprecipitating RASSF1A against NOD2 and the downstream signaling protein involved in NOD2 signaling upon MDP stimulation. (iii) if both abnormal autophagic signaling and abnormal inflammation is triggered in the *Rassf1a*$^{-/-}$ mice, then treatment with either autophagy inhibitor or RIPK2 inhibitor should alleviate DSS-induced inflammation injury. This can be tested by injecting either 3-MA, autophagy inhibitor, or RIPK2

inhibitor-1 in *Rasssf1a*$^{-/-}$ mice upon DSS administration, and record the survival and inflammation outcome of using these inhibitors. (iv) Finally we aim to validate the importance RIPK2 activity in IBD patients using our proprietary pY474-RIPK2 antibody as a surrogate marker for active RIPK2 (Figure 10).

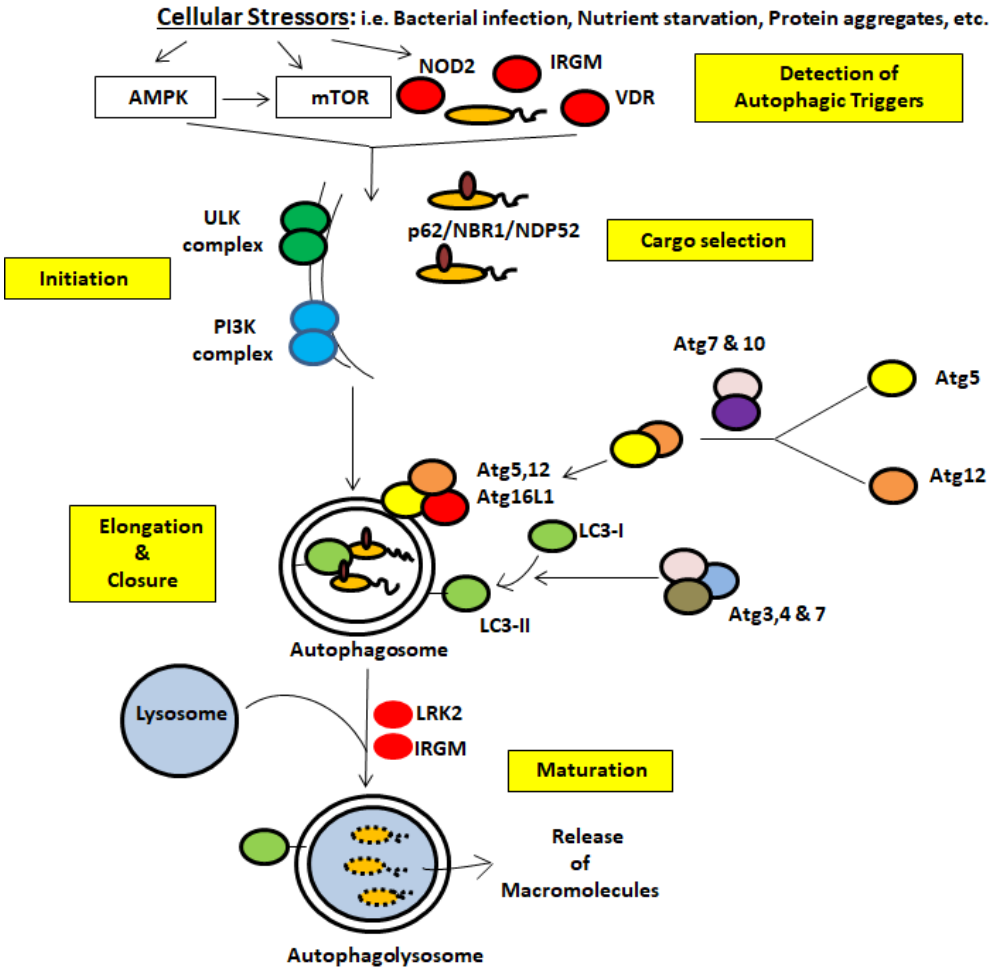


Figure 9: Autophagy signaling pathway: Autophagy is activated by several cellular stressors to activate molecules responsible for autophagy initiation such AMPK, NOD2, etc. Once autophagy is activated autophagosome will encapsulate the cytoplasmic component. The cytoplasmic component within autophagosome will be degraded through lysosome fusion forming autophagolysosomes. Autophagy is divided into four steps including, initiation, elongation, cargo selection, and maturation. These steps are highly regulated and involve many activators and regulators. Proteins in red are GWAS identified genes that associated with IBD [(203)].

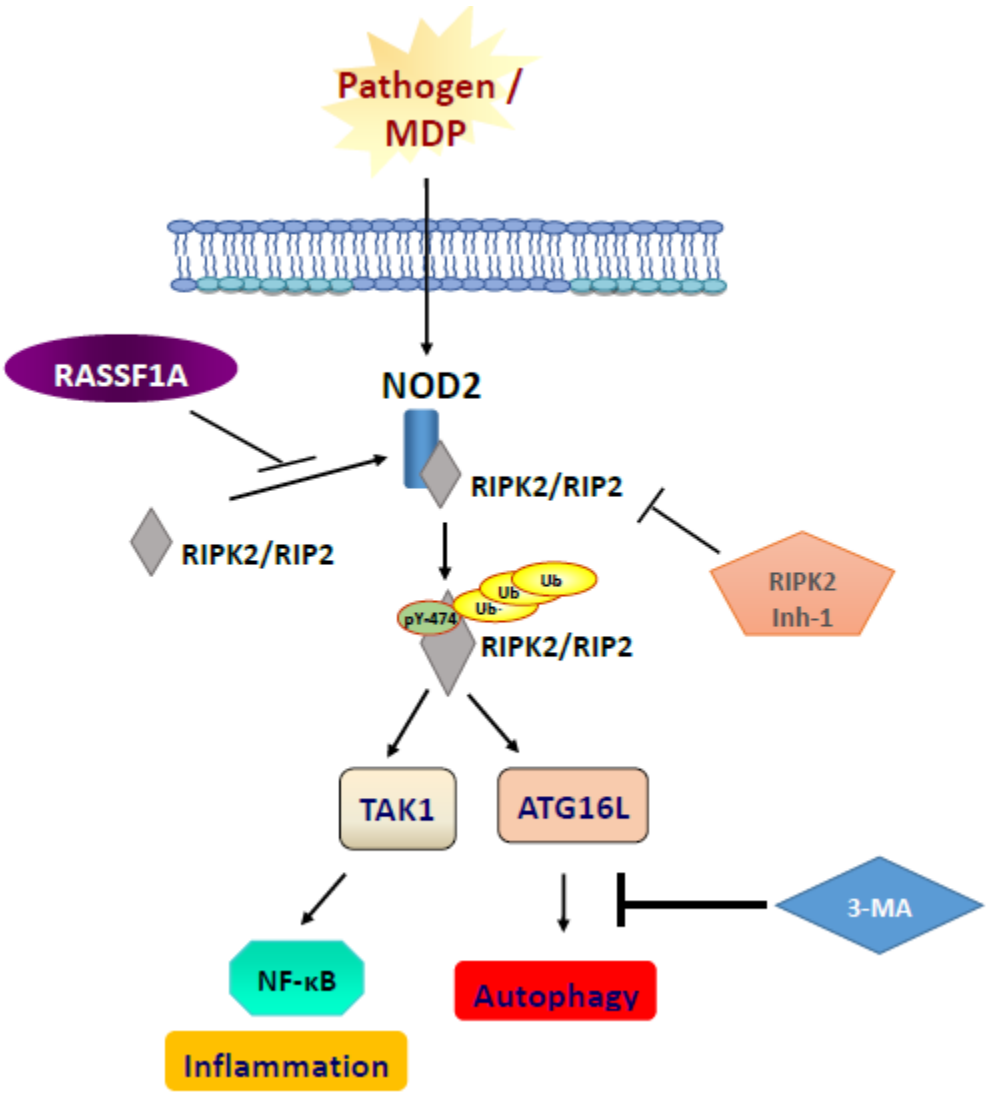


Figure 10: Schematic diagram of RASSF1A on regulating NOD2 signaling pathway: Upon NOD2 sensing of pathogens or MDP conformational changes occur allowing RIPK2 to bind to a NOD2 receptor such binding is restricted by RASSF1A. RIPK2 associates with NOD2 and gets activated by phosphorylation of tyrosine 474 and by K63 ubiquitination. RIPK2 inhibitor-1 downregulates the activation of RIPK2. Through RIPK2 activation inflammation (NFκB) and autophagy (ATG16L1) gets activated. The autophagy signaling pathway is inhibited 3-Methyladenine (3-MA) (Adapted from Dr. Shairaz Baksh).

2 Chapter Two-Materials and Methods

2.1 Materials and buffers:

All chemicals were used according to the manufacturer's specifications as well as by the protocols set out by the Environmental Health and Safety of the University of Alberta and work Hazardous Materials Information System (WHMIS).

2.1.1 Chemicals, Reagents and other Materials:

Acrylamide (Invitrogen 15512-023), Ammonium persulfate (APS) (Biorad 161-0700), Aprotonin (Fisher BioReagents BP2503-10), β mercaptoethanol (Sigma Aldrich M6250), DMEM medium (Fisher Scientific 11965-092), Dextran sulphate (DSS) (36 000-50 000 MW: MP Biomedicals 160110), DMSO (Dimethylsulfoxide) (Fisher Scientific BP2311), Ethanol (Commercial Alcohols P016EAAN), Enhanced Chemiluminescence (ECL) (Homemade), Ethylenediaminetetraacetic acid (EDTA) (Millipore EX0539-1), Ethylene glycol tetra acetic acid (EGTA) (Calbiochem 324626), Glycine (Fisher BioReagents BP3815), Glycerol (Anachemia 43567-360), Hydrochloric acid (Fisher Chemical A144C-212), MDP (Sigma A9519), Methanol (Fisher Chemical A452), Polyvinylidene Fluoride PVDF membrane (Millipore) , PEI (Polysciences, USA 23966-1), PMSF (Thermo Scientific/ Pierce 36978), Protein G Sepharose (GE Healthcare), Sodium dodecyle sulphate (SDS) (BioRad 161-0302), Tris (Invitrogen 15504-020), Trypsin (Fisher Scientific 27250-018), T-PER (Thermo Scientific/ Pierce 78510),Tween 20 (Fisher Bioreagents Whatman Chromatography paper Fisher Scientific BP337) and Z-fix (Anatech 170).

2.1.2 Buffers:

4x separating buffer (1.5M Tris, pH 8.7 and 0.4% SDS), 4x stacking buffer (0.5M Tris, pH 6.8 and 0.4% SDS), 4x SDS-Page loading Buffer For 50mL (13ml Glycerol, 2.5g SDS, 8.7mL of 1M Tris/HCL pH 6.8 and 10mg Bromophenol blue), 10x SDS running buffer (For 1L: 30.2g Tris, 144g Glycine and 10g SDS), SB lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM MgCl2, 1.5 mM EDTA, 0.5% Triton X-100, 20 mM β-glycerolphosphate, 100 mM NaF and 0.1 mM PMSF), 1x wet transfer buffer (For 4L: 12g Tris, 57.6g Glycine, and 800mL methanol and then bring to volume with water), semidry transfer buffer (50mM Tris, 380mM Glycine, 0.1% SDS and 20% methanol), phosphate Buffered saline (PBS) (137mM NaCl, 2.7mM KCL, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4 and pH 7.4) and stripping buffer (52mm Tris pH 6.8 and 2% SDS)

2.2 Antibodies:

2.2.1 Primary antibodies:

Mouse anti-HA (in-house produced, 2µg/µl), Rabbit anti -NOD2 (Santa Cruz sc-30199, 200 µg/ml), Rabbit anti-RIPK2 (Santa Cruz sc-22763, 200 µg/ml), Rabbit anti-pY 474 RIPK2 (125) (In-house made, 3µg/µl), Rabbit anti-p62 (Enzo scientific BML-PW9860-0100, 2µg/µl), Anti-Human PCNA (Caltag Laboratories- PC10, 100 µg/ml), Mouse anti-Ubiquitin (Santa Cruz sc-8015, 200 µg/ml), Rabbit anti-ERK1 (Santa Cruz sc-93, 200 µg/ml) and Rabbit anti-ERK2 (Santa Cruz sc-154, 200 µg/ml)

2.2.2 Secondary antibodies:

Anti-mouse IgG Horseradish Peroxidase-Linked Whole antibody (dilution 1:10000, GE Healthcare UK) and Anti-Rabbit IgG Horseradish Peroxidase-Linked Whole antibody (dilution 1:10000, GE Healthcare UK).

2.3 Drugs:

3-Methyladenine (3-MA): Purchased from Santa Cruz (sc-205596) and autophagy inhibitor, 3-MA was dissolved in sterile water to prepare a stock solution (3mg/ml). RIPK2 inhibitor-1 was purchased from MolPort (MolPort-016-359-762) and dissolved in 100% DMSO to prepare a stock solution (30mg/ml).

2.4 Cell line:

HCT116 (human colon cancer cells) were obtained from Dr. Bert Vogelstein and maintained in RPMI medium respectively plus 10% bovine growth serum (BGS) and incubated in a 37^0C with a 5% containing CO2 incubator. Transfections were carried out using the linear 25 kDa polymer, polyethyleneimine (PEI) obtained from Polysciences, USA (Catalog #23966-2). PEI transfections were carried out by mixing PEI/DNA in a ratio of 4µl PEI/1 µg DNA [(80)].

2.5 Mice:

All animal experiments/husbandry have been approved and follow the guidelines of the Canadian Animal Care and Use Committee and the animal ethics board at the University of Alberta (permit numbers # AUP 219 and AUP 218). *Wild-type, Rassf1a$^{+/-}$ and Rassf1a$^{-/-}$* were (10-14 weeks old) on the C57BL/6 background [(80)]. *Rassf1a$^{+/-}$ and Rassf1a$^{-/-}$* were generated by Dr. Shairaz Baksh using the *Cre recombinase* (Cre)/loxP system. *Rassf1a$^{-/-}$ NOD2$^{-/-}$ and NOD2$^{-/-}$* on the C57BL/6-129 and B6.129S1 background respectively. *Il10$^{-/-}$* mice were (less than 12 weeks old) on the 129S1/SvlmJ background and were obtained from Madsen Lab, University of Alberta or purchased from Jackson laboratories.

2.6 Methods:

2.6.1 Innate immunity analysis:

Animals were administered 3% w/v DSS (#160110, the molecular weight of 36000-50000, MP Biomedicals) in the drinking water for seven days followed by recovery for seven days. They were monitored for: piloerection, bloatedness, tremors, lack of movement, rectal bleeding and weight loss (all on a scale of 0–5 with 5 being very severe) [(204)]. Animals were euthanized once rectal bleeding became grossly apparent. For weight loss, a score of 0 for no weight loss, 1 if, 5% loss, 2 for 5– 10% loss, 3 for 10 – 15% loss, 4 for 15 – 20% loss and a score of 5 for ≤ 25% loss. Disease activity indices (DAI) were the sum of all individual scores [(80)]. All animals were 10 – 12 weeks of age or 25 g in body weight were administrated with 3%DSS at the beginning of the experiment.

2.6.2 Isolation of bone marrow-derived macrophages:

Mice were euthanized by CO_2 asphyxiation and sprayed down with 70% ethanol before dissection. Femurs were dissected out transferred to a sterile petri dish containing DMEM media with 10% bovine growth serum (BGS). BMDM were flushed from femurs in DMEM media and the resulting cell suspension filtered through a 4 μm cell strainer before centrifugation at 16 000 g for 5 min. After pouring off the supernatant, the pellets were washed in 1 mL of sterile PBS, and transferred to a 1.5 mL eppendorf tube before final centrifugation at max speed for 1 min to pellet cells. Supernatant was removed and the dry pellet was stored at -80°C for future use

2.6.3 Cardiac puncture and collection of serum samples:

Cardiac puncture was used to obtain whole blood samples from mice at the time of harvest as a terminal procedure according to the SOP SB005 and standard practice. Collected whole blood was placed immediately into a 1.5 mL microtube on the ice and allowed to coagulate for 1.5 hours.

Samples were then spun in a 4°C microcentrifuge at 16 000g for 10 min. Cleared serum supernatant was collected and stored at -80°C for cytokine analysis.

2.6.4 Tissue Isolation Procedures**:**

Tissue samples collected were for molecular analysis. Samples intended for nucleic acid analyses were immediately submerged in RNA-later post excision and allowed to incubate at 4°C for 24 hours to fully permeate the tissues. At this point samples were removed from RNA-later and stored at -80°C until use. RNA was isolated from colon tissue using the Qiagen All Prep DNA/RNA spin column nucleic acid extraction kit (Qiagen 80204) according to the manufacturer's directions. Nucleic acid concentration was determined using the Thermo Scientific Nanodrop. Colon samples intended for molecular protein analysis were excised from the mouse, flushed with 1X PBS to remove fecal matter, and immediately submerged in 1 mL ice-cold RIPA buffer with fresh 0.1% aprotinin (Fisher BioReagents BP2503-10), 0.2% phenylmethylsulfonyl fluoride (PMSF) (Thermo Scientific/ Pierce 36978), 0.1% sodium pyrophosphate ($Na_4O_7P_2$), 1% sodium orthovanadate (Na_3VO_4) (Calbiochem 56740), and 0.2% SL protease inhibitor cocktail in a 2 mL eppendorf tube . Samples were then homogenized using a Fisher PowerGen handheld homogenizer and centrifuged at 4°C at max speed for 10 min. The soluble protein supernatant was collected and stored at -80°C until use. Protein concentration was determined using a Bradford protein assay.

2.6.5 Crypt cell isolation:

Colons were flushed with cold 1× PBS, cut open longitudinally and then soaked in 1X PBS with gentle shaking for 20 minutes. The colons were cut into small pieces and incubated with 0.04% sodium hypochlorite for 30 min with gentle shaking, followed by incubation at room

temperature for 30 minutes in a solution containing 1X PBS/1 mM EGTA/1 mM EDTA with shaking. Cells were then dislodged by pipetting the tissue up and down using a 25 mL serological pipette until the solution became cloudy. The supernatant was removed (containing the crypt cells) and centrifuged at 3000 rpm for 10 minutes to collect the crypt cells followed by nuclear extraction.

2.6.6 Preparation of nuclear extracts:

Nuclear extracts were obtained from crypt cell and BMDM pellets using the NE-PER nuclear and cytoplasmic fraction kit from Pierce Scientific according to the manufacturer's directions. Purity of the resulting nuclear and cytoplasmic fractions was confirmed using Western blotting for β-tubulin (Sigma-Aldrich T5201, 1:1000 IB) and lamin B (Invitrogen 33-2000, 1:500 IB), with the cytoplasmic fraction expected to have tubulin and no lamin B, and the nuclear fraction expected to have plentiful lamin B and little to no tubulin. Fractions were stored in 1.5 mL eppendorf tubes at -80°C until use.

2.6.7 Tissue histology and immunohistochemistry:

Tissue Histology: Colon samples were isolated, fixed in Z-fix (Anatech 170) and paraffin-embedded. All inflammation scores were obtained utilizing blinded scoring by a gastrointestinal pathologist (Dr. Aducio Thiesen) based on infiltration of enterocytes, neutrophils, lamina propria cellularity, crypt structure and epithelial hyperplasia (scored as 0-2 where 2=maximal injury) [(80)].

Immunohistochemistry. Immunohistochemistry and hematoxylin and eosin (H&E) staining were carried out using standard techniques. Formalin-fixed, paraffin-embedded sections were de-paraffinized and re-hydrated. Antigen retrieval was done by boiling in sodium citrate (Fisher Chemical S279) buffer. Endogenous peroxidase activity was quenched with 3% H_2O_2.

Sections were blocked in 2% BSA + 2% donkey serum for one hour at room temperature and incubated in primary antibody as indicated overnight at 4°C. Sections were incubated in 1:500 biotinylated secondary antibody for 1 hour at room temperature and signal amplification and detection was done using the VECTASTAIN Elite ABC Kit (Vector Laboratories PK-6100) and the Metal Enhanced DAB Substrate Kit (Thermo Scientific/ Pierce 34065). Counterstaining was done using Harris' modified hematoxylin.

2.6.8 Immunoblotting:

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins from protein samples using standard techniques [(80)]. Proteins were transferred to methanol activated polyvinyl difluoride (PVDF) membrane using a wet transfer for large proteins (100 V for 1.5 hours) and semi-dry transfer for proteins smaller than 37 kDa (460 mA for 2 hours) [(80)]. Where fold changes are indicated, all samples have first been normalized to their respective ERK1/2 (Santa Cruz Biotechnology, sc-93 and sc-154) loading controls, and then fold changes calculated as compared to baseline wild-type animals. Anti-HA (1:500, in-house produced), Anti -NOD2 (1:200, Santa Cruz, sc-3019), Anti-ATG16L1 (1:1000, Cell signaling, D6D5), Anti-RIPK2 (1: 1000, Santa Cruz, sc-22763), Anti-p62 (1:1000, Enzo scientific, BML-PW9860-0100), Anti-pY474 RIPK2 (1:1000, in-house produced) and Anti-pS176 RIPK2 (1:500, Cell Signaling, 14397S).

2.6.9 Enzyme-linked immunosorbent assay (ELISA)**:**

Mouse Cytokine Array/Chemokine Array 32-Plex Panel (Eve Technologies, Calgary, AB). The multiplexing technology used by Eve technology is based on color-coded polystyrene beads. The beads are created by using a different concentration of red and infrared fluorophore dyes to

create sets of colored beads. These beads are combined into one well and each bead is coupled with a specific antibody for a specific protein. When the samples have added the proteins of interest will bind to its antibody forming a plex. A biotinylated secondary antibody conjugated with streptavidin-phycoerythrin are added to the mixture, and the mixtures are then analyzed by bead analyzer containing dual-laser system and a flow-cytometry system. The amount of conjugate detected by the analyzer is proportional to the amount of analyst. The results are quantified according to a standard curve.

2.6.10 NFκB electromobility shift assay (EMSA):

Briefly, duplex DNA specific for the NFκB binding site was end-labeled with [γ-^{32}P] ATP by using T4 polynucleotide kinase, purified using a G-50 Sephadex column (Roche) and allowed to associate with 4 μg of the nuclear extracts containing NFκB for 30 min at room temperature. The oligonucleotide was AAATGTGGGATTTTCCCATGA for crypt cell NFκB analysis (the NFκB binding site from the IL-6 promoter) and TCAGAGGGGACTTTCCGAGAGG for BMDM NFκB analysis (the NFκB binding site from the IL-8 promoter). DNA/protein complexes were then separated by non-denaturing gel electrophoresis, dried onto Whatman filter paper and autoradiographed. Nuclear extracts were prepared using NE-PER (ThermoFisher Scientific) as per manufacturer's instructions). Binding buffer was 100 mM Tris-HCl pH 6.5, 500 mM KCl, 1.2 mM EDTA, 12 mM DTT, 20% glycerol and 1 μg Salmon Sperm DNA. The purity of fractions was confirmed with anti-β-tubulin (cytosolic fraction) or anti-lamin B (nuclear fraction) [(205)].

2.6.11 NFκB Gene Reporter Luciferase Assay:

Dual-Luciferase Reporter Assay System (DLR assay system, Promega, E1910) was used to perform dual-reporter assays on NFκB Luciferase and Renilla Luciferase (internal control).

Briefly, cells were equally seeded at a density of 3 x 104 in 6-well plates and allowed to attach for 24 hrs. Before transfection, cells were washed with serum-free media three times. Dual transfection was carried out using PEI adding 3 ug of NFκB Luciferase construct and 60 ng of Renilla Luciferase construct. After 24hr transfection, cells were treated with the different drugs for 24-36 hrs. Cells were then lysed using the passive lysis buffer provided by the kit for 30 mins on ice.

The lysate was spun down for 8 minutes at 10,000 rpm, and 20 ul of cell lysate were transferred to 96-well plate. Luciferase assays were analyzed based on the ratio of Firefly/Renilla to normalize cell number and transfection efficiency.

2.7 Statistics:

Statistical analyses were performed using one-way or two-way ANOVA with Tukey or Bonferroni post-hoc tests respectively, or Students t-test (two-tailed), as indicated using the GraphPad Prism 5 software. The error bars represent SD and the significance are represented in the legends means (ns) $P > 0.05$, (*) $P \leq 0.05$, (**) $P \leq 0.01$ and (***) $P \leq 0.001$.

3 Chapter Three - Genetic loss of NOD2 protects *Rassf1a*$^{-/-}$ mice from DSS-induced acute Inflammation Injury

3.1 Rationale:

In the publication of Gordon et al., (2013), it was demonstrated that RASSF1A could associate with Toll-like receptors (TLR), a pathogen recognition receptor, to restrict nuclear factor-κB (NFκB) activation. The association of RASSF1A with TLRs supports the importance of RASSF1A in modulating inflammation and thus the loss of RASSF1A showed abnormal stimulation of NFκB with subsequent inflammation. As previously mentioned *Rassf1a*$^{-/-}$ mice upon DSS administration mimicked clinical symptoms similar to human colitis including increased intestinal permeability, enhanced cytokine/chemokine production, elevated NFκB activity, severe colonic epithelial cell injury and less than 20% survival were observed in these mice.

Furthermore, changes in p62 expression level were observed in *Rassf1a*$^{-/-}$ with DSS when compared to wild-type. Since p62 acts as a selective autophagy receptor for ubiquitinated protein aggregation and ATG16L1 regulates p62 level via autolysosomal pathways. It expected that NOD2 might have a role in mediating inflammation in *Rassf1a*$^{-/-}$ since NOD2 and ATG16L1 physically associate with each other for bacterial clearance upon pathogen invasion in the cells. The association of RASSSF1A with NOD2 may play a role in restricting NOD2 signaling pathway leading to both autophagy and NFκB activation reduction. To test the first objective if NOD2 is the gene responsible for inducing inflammation in RASSF1A knockout mice.

3.2 Results:

3.2.1 *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ mice showed improve survival against DSS

To test if NOD2 is inducing inflammation in the absences of RASSF1A. *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ and *Nod2*$^{-/-}$ mice were administrated with 3% DSS in drinking water for seven days followed by fresh water for recovery for another seven days. Mice were monitored for signs of colitis including weight loss, rectal bleeding, piloerection, bloatedness, hunching, movement, tremors and stool

consistency. The phenotypic characteristics were assigned a score of 0–5 with five being very severe and animals were euthanized once rectal bleeding became grossly apparent. Interestingly, both *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ and *Nod2*$^{-/-}$ mice demonstrated improved survival rate (Figure 11 A) and reduced disease activity index when compared to DSS treated *Rassf1a*$^{-/-}$ mice (Figure. 11 B). This demonstrates that NOD2 is inducing inflammation in *Rassf1a*$^{-/-}$ mice upon DSS administration and the loss of NOD2 induced recovery and survival in these mice.

3.2.2 The loss of NOD2 showed protection against DSS induced inflammation:

Does the loss of NOD2 protect RASSF1A knockout mice for DSS induce inflammation? We answered that by investigating the inflammation outcome of these genotypes upon DSS administration, on day eight the mice were euthanized and both blood and colon sections were harvested for analysis. *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ and *Nod2*$^{-/-}$ showed low histopathological score (Figure 12 A), retain of tissue architecture (Figure 12 B) and high neutrophil activity (Figure 12 C) when compared to *Rassf1a*$^{-/-}$ mice treated with DSS. Both *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ and *Nod2*$^{-/-}$ showed low cytokine production (Figure 12 E) and increased crypt cells proliferation indication of recovery (Figure 12 D). Indicating that the loss of NOD2 in RASSF1A knockout mice showed reduce inflammation and induce recovery. We then wanted to analyze p62 in these mice genotypes since we already found alteration in p62 expression level in *Rassf1a*$^{-/-}$ mice when compared to WT.

3.2.3 p62 expression level analysis in colon lysates

p62 protein serves to link ubiquitinated proteins to the autophagic machinery for degradation by lysosomes and p62 gets degraded by autophagic signaling [(206)]. p62 is considered as a marker to study autophagy regulation since the accumulation of p62 occurs when autophagy

is inhibited. Autophagic flux term indicates, autophagosome formation, a fusion of the autophagosome with lysosome and degradation of autophagic substrates inside the lysosome. Some studies consider p62 as a reliable indicator to investigate autophagic regulation [(207)]. Colon lysates from DSS treated mice were harvested for autophagy biomarker analysis using immunoblotting. DSS-treated *Rassf1a* $^{+/-}$ mice revealed decreased levels of p62 when compared with *Nod2*$^{-/-}$ and *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ treated with DSS, indicating the increase in the autophagic signaling in *Rassf1a* $^{+/-}$ (Figure 13 A-B). *Nod2*$^{-/-}$ mice showed accumulation of p62 meaning that autophagy is downregulated, but for *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ mice, they showed a slight reduction in autophagy due to the loss of RASSF1A showing its role in regulating autophagy.

3.3 Conclusion:

These results indicate a robust role for the NOD2/RIPK2 pathway in promoting inflammation injury in responsive to DSS in the *Rassf1a*$^{-/-}$ mice and the loss of NOD2 in mice showed a protective effect against inflammation upon DSS administration when compared to *Rassf1a*$^{-/-}$. Indicating that inflammation might be regulated by NOD2 activity in the absences of RASSF1A leading to poor survival and recovery in *Rassf1a*$^{-/-}$ mice when compared to *Rassf1a*$^{-/-}$ *Nod2*$^{-/-}$ and *Nod2*$^{-/-}$. Since autophagy is regulated upon NOD2 activation, the loss of NOD2 in both *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ and *Nod2*$^{-/-}$ mice showed accumulation of p62 when compared to *Rassf1a*$^{-/-}$ mice. But *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ when compared to *Nod2*$^{-/-}$ showed low p62 expression level indicating that a different pathway is regulating p62 rather than RASSF1A and NOD2. RASSF1A showed to be important in regulating NOD2 induced activation as it is observed when comparing the DSS outcome of WT, *Nod2*$^{-/-}$ and *Rassf1a*$^{-/-}$ mice. Further experiments need to be done to confirm autophagy regulation in these mice genotypes and these experiments will be discussed in the future experiments section.

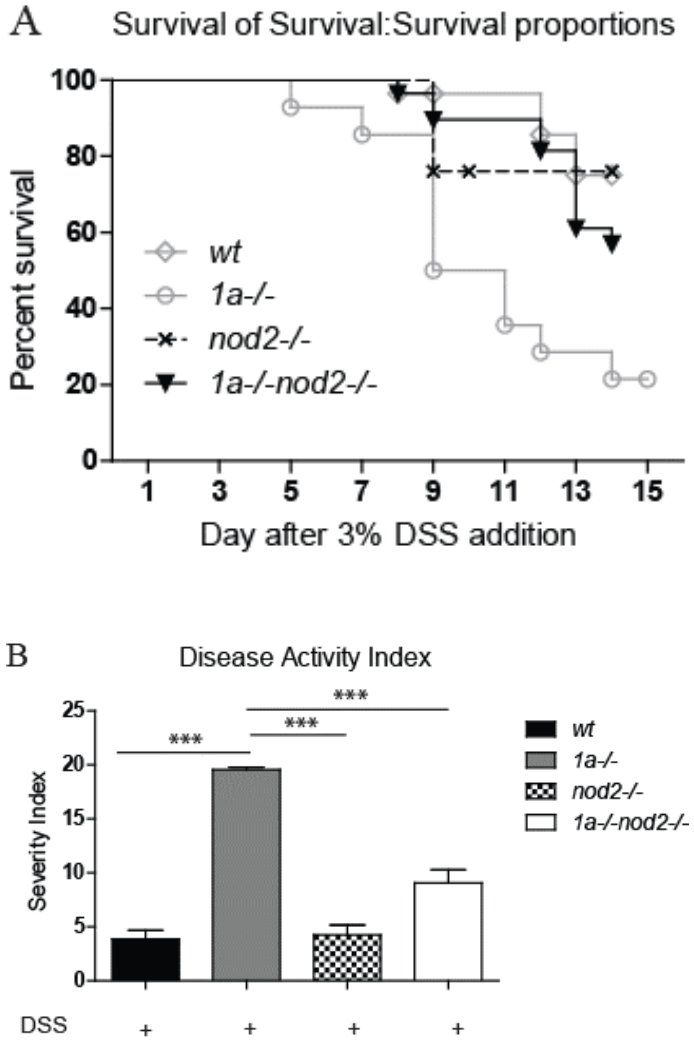
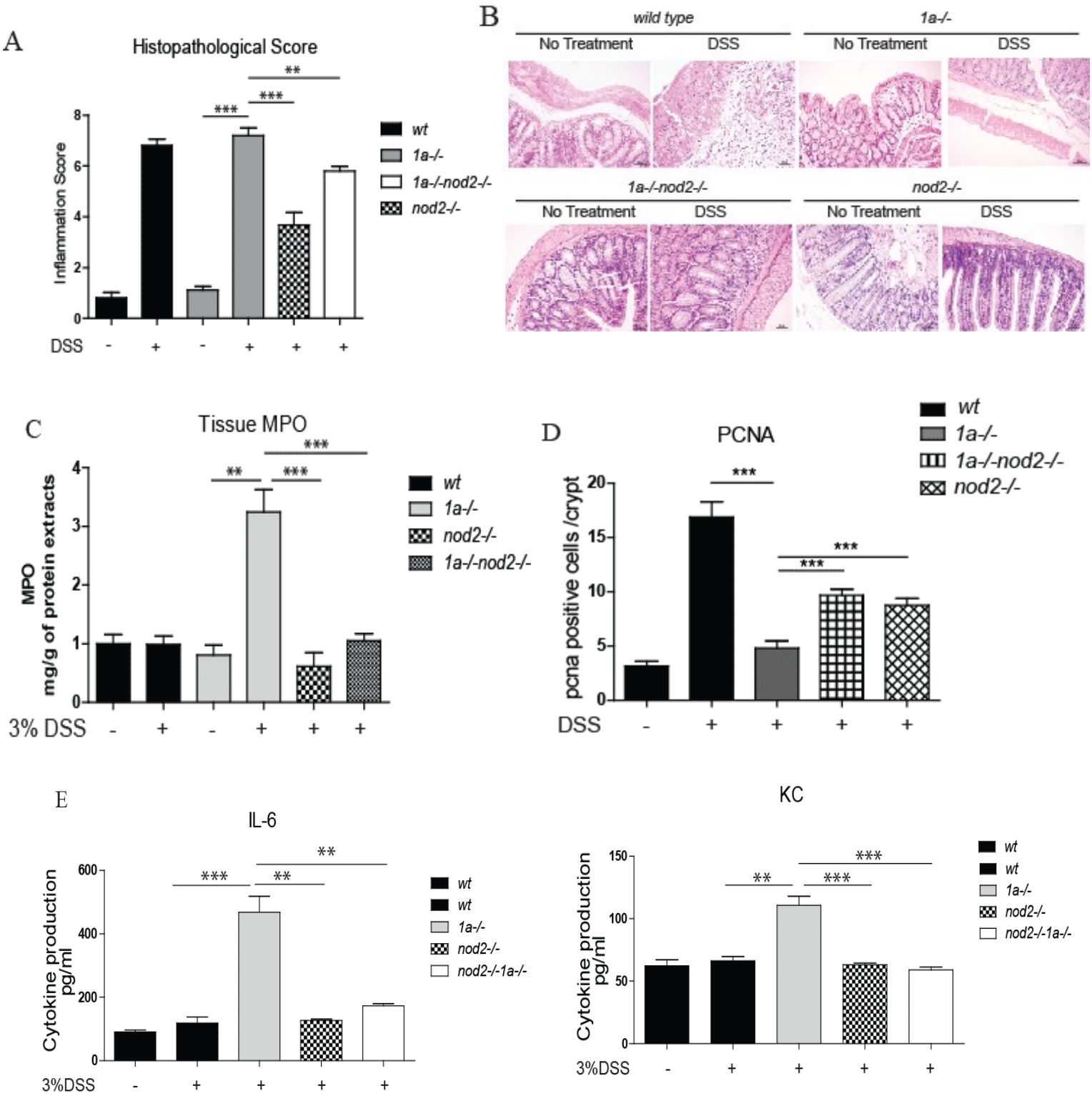


Figure 11: *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ mice showed improve survival against DSS. Mice were subjected to 3% DSS solution followed by day seven replacements with regular water to allow for recovery. (A) A Kaplan-Meier curve is monitoring % survival following DSS treatment. n =14-43. p-value is < 0.0001 WT vs. *Rassf1a*$^{-/-}$ and *Rassf1a*$^{-/-}$ vs *Nod2*$^{-/-}$ and *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ p-value = 0.0053 (**) (Data provided by Mohammed El-Kalla and panel A figure prepared by D. Shairaz Baksh). (B) Disease activity index (DAI) of these mice were monitored following DSS treatment. P value <0.0001 (***), n = 11-25.


A
Histopathological Score
Inflammation Score
DSS
wt
1a-/-
1a-/-nod2-/-
nod2-/-
B
wild type
1a-/-
No Treatment
DSS
1a-/-nod2-/-
nod2-/-
C
Tissue MPO
MPO
mg/g of protein extracts
3% DSS
D
PCNA
pcna positive cells /crypt
E
IL-6
Cytokine production pg/ml
3%DSS
KC
1a-/-nod2-/-1a-/-

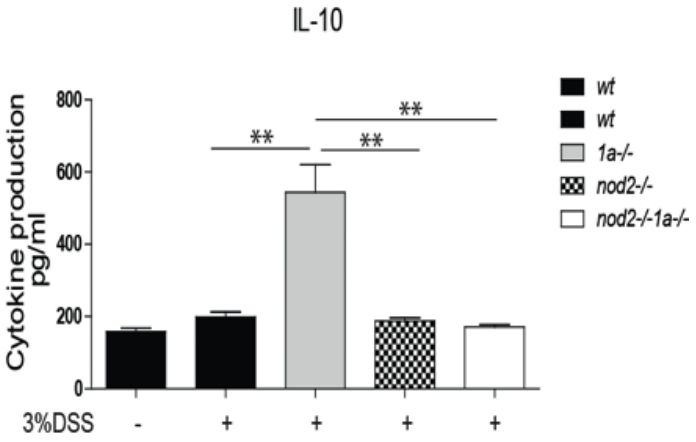


Figure 12: The loss of *Nod2* showed protection against DSS induced inflammation. (A) Tissue MPO activity as determined by ELISA. P value are 0.0037 (**), 0.0003 (***) and <0.0001 (***). n=4-17. (B) The longitudinal cross-section of the descending colon stained with H&E and figures of the section was taken using an AxioCam ERc 5s camera at X400 magnification (Zeiss AX10 Observer.Z1 inverted microscope; Carl Zeiss Canada Ltd., Toronto, ON, Canada). (C) Tissue MPO activity as determined by ELISA. P value are 0.0037 (**), 0.0003 (***) and <0.0001 (***). n=4-17. (D) PCNA staining was measured for these genotypes that were observed. P value <0.0001 (***), n = 6-22. (E) Blood serum of the treated animals was analyzed for cytokine production using ELISA. P value for *Il-6* are 0.0002 (***), 0.0025 (**) and 0.0044 (**). n=3-5. P value for KC are 0.0002 (***) and 0.0019 (**). n=3-5. P value for *Il10* are 0.0017, 0.0022 and 0.0030 (**). n=4-7.

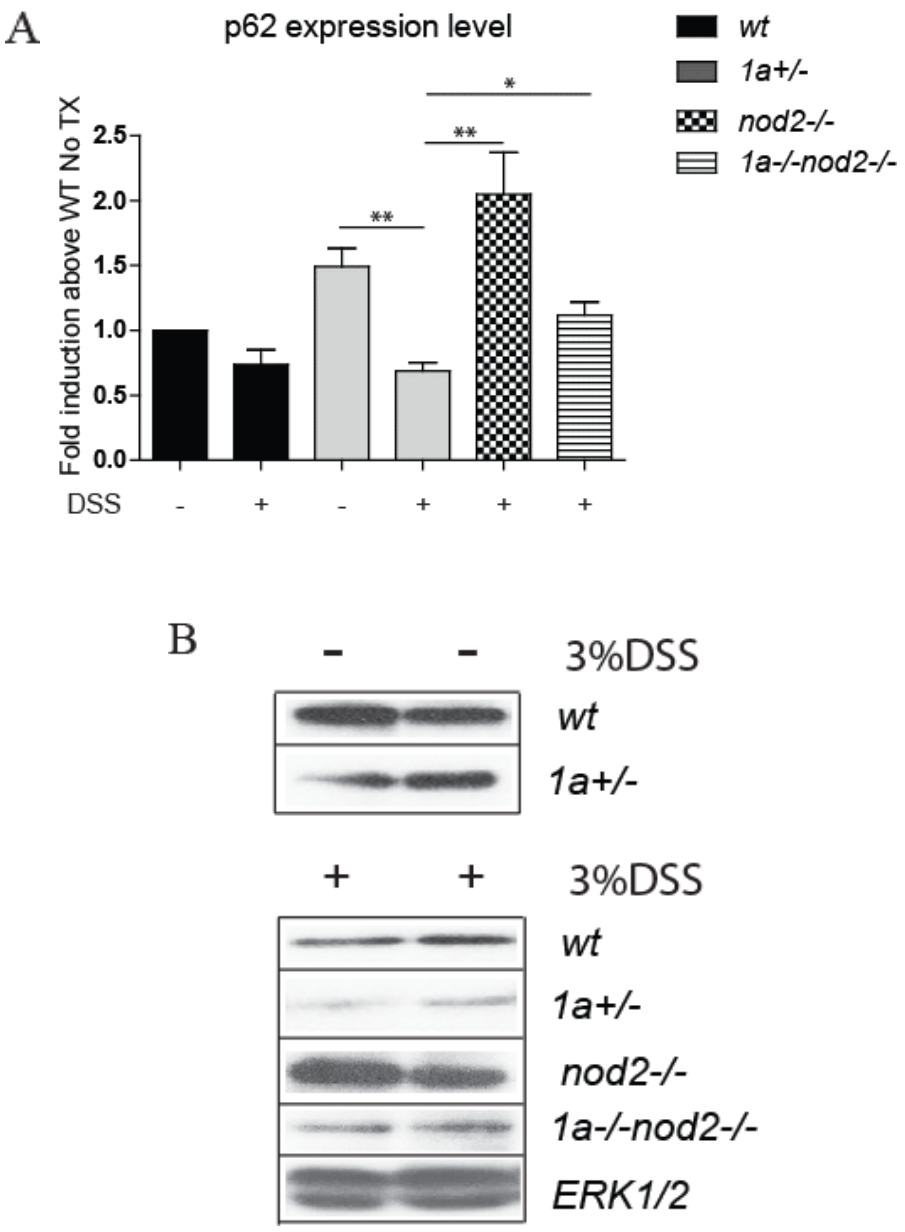


Figure 13: p62 expression level analysis in colon lysates. (A) p62 expression level was measured from the following colon lysates through using p62 antibody following the proper concentration. P values are 0.0022 (**), 0.0046 (**) and 0.0137 (*). n= 3-5. (B) Analysis for p62 protein expression level following *in vivo* DSS insult in colon lysate.

4 Chapter Four-RASSF1A regulates NOD2 signaling pathway

4.1 Rationale:

NOD2 is a pathogen recognition receptor found in the cytoplasm and required to respond to the certain bacterial invasion. Upon NOD2 activation with its specific ligand, muramyl dipeptide (MDP) downstream receptor-interacting serine-threonine kinase 2 (RIPK2) gets recruited and both ATG16L1 (to drive autophagy) and NF-κB gets promoted [36, 139]. RIPK2 is a crucial protein to NOD2 activation, and RIPK2 deficient showed no response to NOD2 ligand [144].

Previously we demonstrated robust associations of RASSF1A with TLR2 and TLR4 resulting in inhibition of cytokine production in LPS stimulated colon cancer cultured cells overexpressing RASSF1A [80]. RASSF1A association with these complexes function to restrict TLR driven activation of NFκB signaling [80]. We also observed that upon losing NOD2 in *Rassf1a*$^{-/-}$ mice the inflammation outcome of DSS treated mice was almost similar to the outcome observed in *Nod2*$^{-/-}$ and WT when compared to Rassf1a$^{-/-}$ mice. Therefore, we decided to explore the ability of RASSF1A to physically associate with NOD2 and restrict the association of RIPK2 with NOD2 and inhibit activation of NF-κB and /or autophagy after stimulation with MDP. We also wanted to investigate which domain in RASSF1A is necessary for the NOD2 association and the NFκB activity outcome for such association.

4.2 Results:

4.2.1 RASSF1A can physically associate with NOD2 and restrict RIPK2 association with NOD2

To test if RASSF1A physically associate with NOD2 and restrict RIPK2 association with NOD2. We used HCT116 cells which were transfected with/without HA-RASSF1A and were stimulated with MDP (0, 1, 3 and 5 hrs). HCT116 cells do have both NOD2

and RIPK2 endogenously expressed. The cells were harvested, lysed, immunoprecipitated with anti-NOD2 antibody and immunoblotted for NOD2 and RIPK2. Similar to TLR association, we demonstrated RASSF1A association with NOD2 robustly upon MDP addition (Figure 14 A). We also demonstrated that RASSF1A could interfere with the association of RIPK2 with NOD2, suggesting that RASSF1A can negatively regulate NOD2 pathway after stimulation with MDP to inhibit RIPK2 recruitment to NOD2 (Figure 14 B). We speculate that upon DSS treatment the lack of RASSF1A in mice resulted in uncontrolled activation of a NOD2 signaling pathway that might be detrimental to epithelial repair and recovery.

4.2.2 RASSF1A restricts ATG16L1 association with NOD2

We observed a physical association of RASSF1A with NOD2 and restriction of RIPK2 association with NOD2 in the presences of RASSF1A. Our next question was: does RASSF1A also restrict an ATG16L1 association with NOD2. HCT116 cells were transfected with/without *HA-RASSF1A* and were stimulated with MDP (0, 1, 3 and 5 hrs). The cells were harvested, lysed, immunoprecipitated with anti-NOD2 antibody and immunoblotted for ATG16L1. RASSF1A was found to restrict ATG16L1 association with NOD2 upon MDP stimulation. This suggests that the role of RASSF1A in downregulating NOD2 activation by restricting the association of ATG16L1 with NOD2 (Figure 15).

4.2.3 Effect of RASSF1A deletion/mutations on NOD2 Signaling and NFκB Activation

As mentioned previously RASSF1A is tumor suppressor protein having three different domains. These domains are important in mimicking RASSF1A function which are RA, SARAH and zinc finger domain. We asked ourselves which domain in RASSF1A is important for

RASSF1A association with NOD2. HCT116 cells were transfected with different deletions and polymorphic mutants of RASSF1A's domain linked to an HA tag. The cells were harvested, lysed, immunoprecipitated with anti-NOD2 antibody and immunoblotted for HA. We observed the importance of the zinc finger domain and Ras-binding domain mutants for association with NOD2 upon MDP stimulation. We also asked ourselves if these deletions and polymorphism are important not only for association with NOD2 but also for regulating NFκB. Using a dual luciferase system where the firefly luciferase construct is under the control of the NFκB binding site of the IL-6 promoter and the Renilla Luciferase as an internal control; some of these truncations and polymorphic mutants showed effectiveness in regulating NFκB activity when RASSF1A losses its association with NOD2 (Figure 16 A-B). This suggests that the zinc finger and Ras domain are not only critical for RASSF1A/NOD2 association but also for downregulating the downstream signaling pathway of NOD2 which is NFκB activity.

4.3 Conclusion:

These results support the role of RASSF1A in restricting NOD2 signaling pathway has previously been shown that upon the loss of RASSF1A in knockout mice NOD2 is responsible for induced inflammation and poor recovery for these mice upon DSS administration. Here we prove that RASSF1A physically associate with NOD2 upon biological response especially with MDP and RASSF1A restrict the association of RIPK2 to NOD2. RIPK2 is a major protein in the NOD2 downstream signaling pathway, and upon RIPK2 activation NFκB gets activated. We observed by using NFκB luciferase activity assay the overexpression of RASSF1A in HCT116 downregulated NFκB activity and that supports the outcome of RASSF1A regulating NOD2 signaling pathway. We also found that RASSF1A regulates another downstream signaling pathway of the NOD2 signaling pathway, which is autophagy by restricting ATG16L1 association -with NOD2. Finally,

we wanted to know which domain is important in facilitating RASSF1A association with NOD2 and by using different truncation and polymorphisms of RASSF1A domain we realized that the zinc finger domain and Ras-binding domain mutants are important for RASSF1A association with NOD2. These truncations and polymorphism not only proved their importance for RASSF1A association with NOD2 but also they confirmed our hypothesis of RASSF1A regulating NOD2 signaling pathway by analyzing NFκB activity which is a biomarker for NOD2 activity.

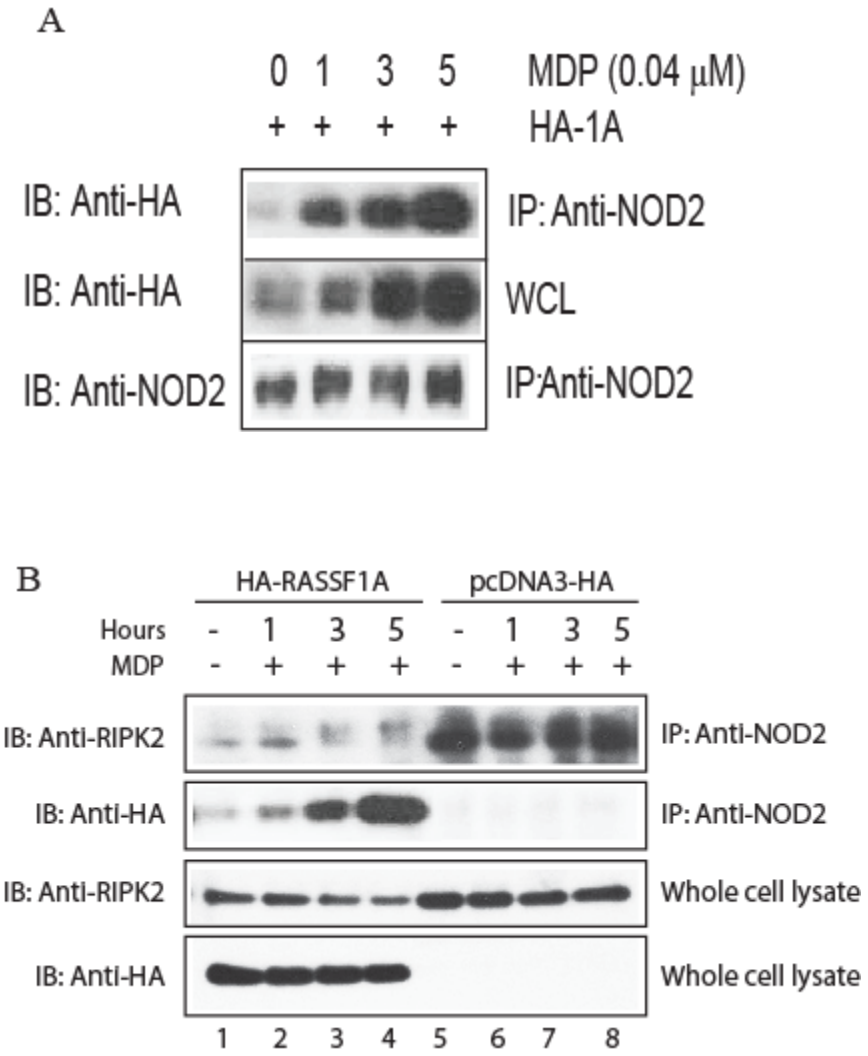


Figure 14: RASSF1A can physically associate with NOD2 and restrict RIPK2 association with NOD2. (A) HCT116 cells were transiently transfected with HA-RASSF1A and stimulated with MDP in different time course. The cells were then lysed with RIPA buffer and immunoprecipitated against NOD2 and blotted against HA (Data courtesy of Shairaz Baksh). (B) HCT116 cells were overexpressed with HA-RASSF1A. The lysates were immunoprecipitated by RIPK2 and blotted against HA and RIPK2 (Data courtesy of Shairaz Baksh).

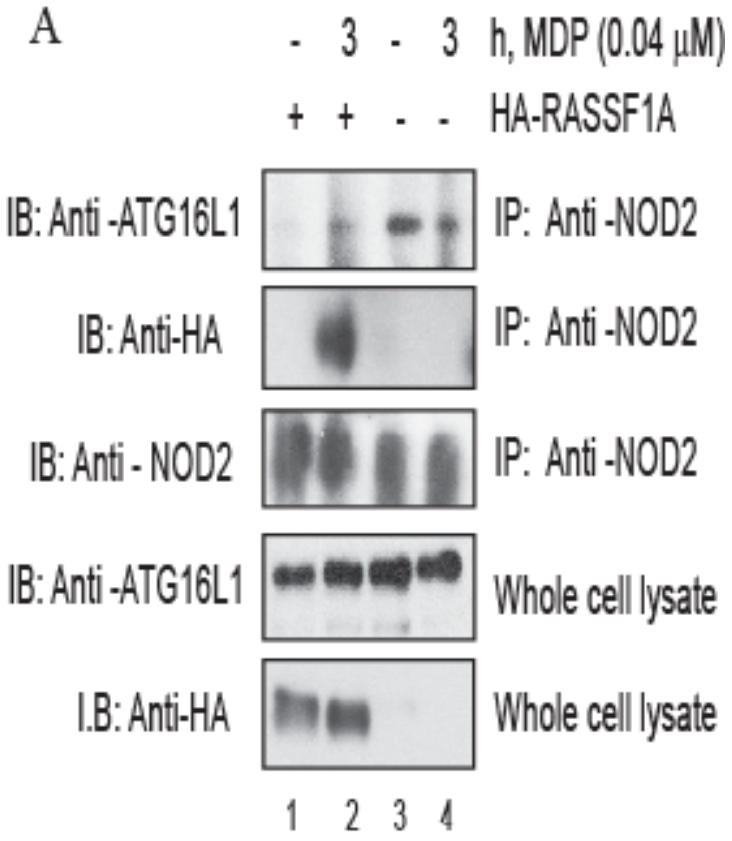


Figure 15: RASSF1A restricts ATG16L1 association with NOD2. HCT116 cells overexpressed with HA-RASSF1A and the lysates were immunoprecipitated by NOD2 and blotted against HA, NOD2 and ATG16L1 (Data courtesy of Shairaz Baksh).

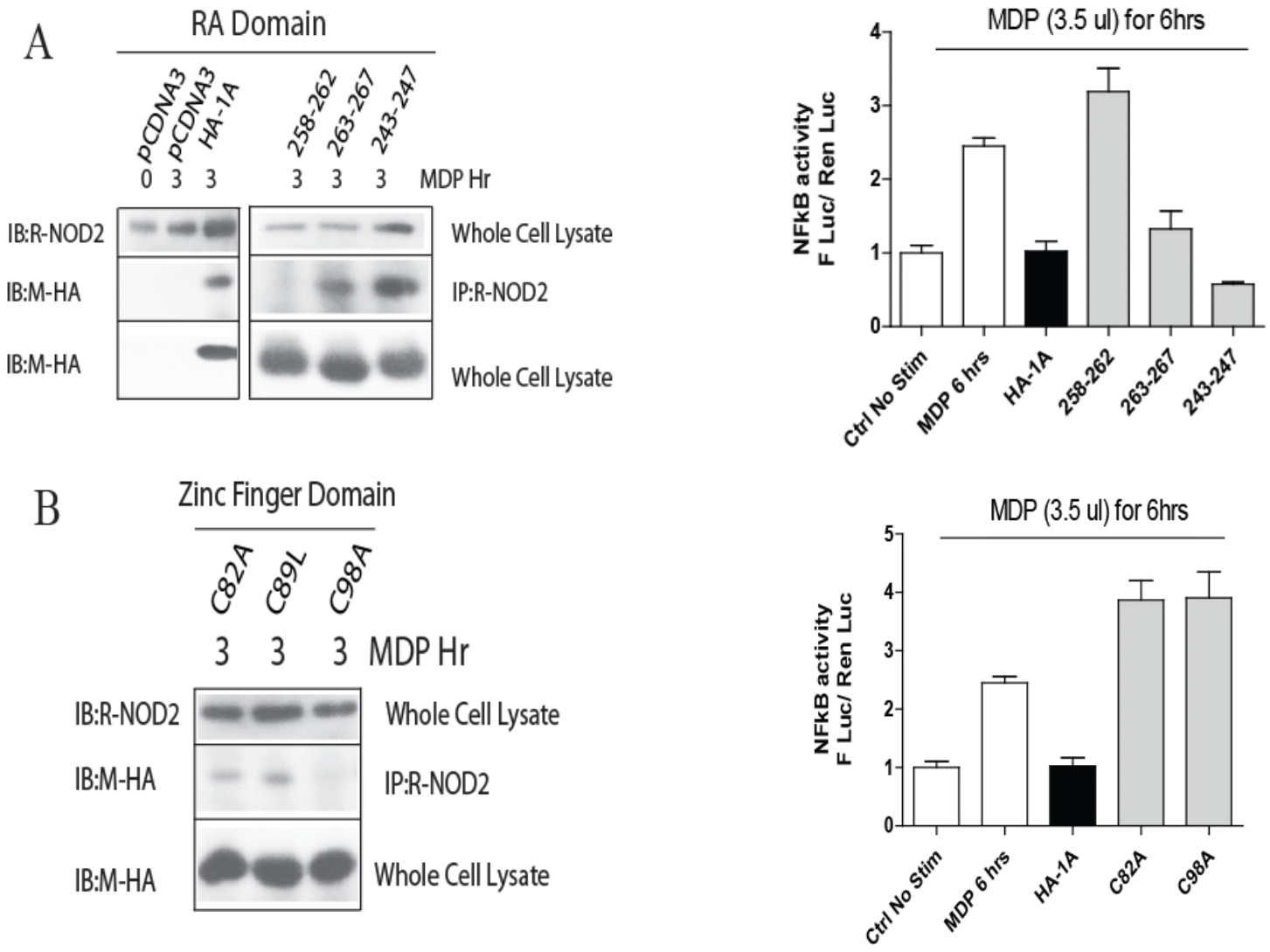


Figure 16: Different mutations and truncations of RASSF1A showed regulation of NOD2 signaling pathway. (A-B) HCT116 is overexpressed with 3µg of RASSF1A constructs and stimulated with MDP for 3hr. Cells were lysed by scraping into 1 ml of 1 X PBS, combined, and lysed in 800 µl of RIPA buffer. The lysates were then immunoprecipitated with 1.2 µg with NOD2 and then blotted against HA. The right panel NFκB activity of the different truncations of Rassf1a and deletions were measured by using NFκB luciferase assay (Worked done by Mohammed Salla).

5 Chapter Five-Inhibition of autophagic signaling protects *Rassf1a*$^{-/-}$ mice from DSS-induced acute inflammation injury

5.1 Rationale:

Autophagy is a normal process in the body that deals with destruction of cells in the body. It maintains homeostasis or normal functioning by protein degradation and turnover of the destroyed cell organelles for new cell formation. Many genes that have either genetic or functional links to autophagy is associated with IBD including NOD2 and ATG16L1 [(168)]. Previously we showed in the absence of RASSF1A p62 expression was downregulated and *in-vitro* study showed that the presences of RASSF1A restricted the association of ATG16L1 with NOD2. Interestingly, both *Rassf1a*$^{-/-}$ *Nod2*$^{-/-}$ and *Nod2*$^{-/-}$ mice showed resistant to DSS induced inflammation, suggesting that the damage from DSS might be through the active NOD2 signaling. We demonstrated that RASSF1A forms a robust association with NOD2 upon MDP stimulation in colon cancer cell line (HCT116), indicating that RASSF1A negatively regulate NOD2 signaling and downregulating both inflammation and autophagy upon MDP stimulation. These data suggest that upon DSS administration, NOD2 activation is induced leading to autophagy regulation in *Rassf1a*$^{-/-}$ mice.

In this chapter we will explore the potential effect of autophagy inhibitors, 3-methyladenine (3-MA) on DSS-treated mice to inhibit the autophagic response that might be detrimental to epithelial repair and healing after inflammation-induced injury. Autophagy inhibitor, 3-MA inhibits early stages of autophagic cascades via inhibition of type III Phosphatidylinositol 3-kinases (PI-3K) [(208, 209)]. We speculate that the pharmacological inhibition of the autophagic response could be a novel therapeutic approach to enhance recovery from inflammation-induced injury and be useful in treating and saving IBD patients from increased risk of getting cancer later in life.

5.2 Results:

5.2.1 Autophagy inhibitor 3-MA showed improve survival of *Rassf1a*$^{-/-}$ upon DSS administration

To answer the question that the pharmacological inhibition of the autophagic response could be a novel therapeutic approach to enhance recovery from inflammation-induced injury. *Rassf1a*$^{+/-}$ mice (10-14 weeks old) were administrated 3% DSS in drinking water for seven days followed by fresh water for recovery for another seven days. Autophagy inhibitor, 3-MA (20 ug/g body weight) was injected by intraperitoneal injections on day 3 and 6. Interestingly, the anti-autophagy drug, 3-MA significantly increased the survival rate of DSS-treated *Rassf1a*$^{+/-}$ mice to >70% when compared with *Rassf1a*$^{+/-}$ mice on DSS which have survival rate about 25% (Figure 17 A). Also, there was a significant decrease in disease activity index indicating that recovery from inflammation symptoms was induced due to the use of 3-MA (Figure 17 B). Therefore, inhibiting the damaging effects caused by autophagy in *Rassf1a*$^{-/-}$ mice using 3-MA showed significant protection from DSS insult.

5.2.2 3-MA protected *Rassf1a*$^{-/-}$ from DSS induced inflammation

To confirm if 3-MA showed significant protection from DSS insult, after treating *Rassf1a*$^{+/-}$ with autophagic inhibitor 3-MA upon DSS administration. These mice were euthanized and both blood and colon were harvested for analyzing the inflammation outcome. We observed retain of tissue structure in the descending tissue sections indicating active repair in the presence (Figure 18 A) as well as increased colon length (Figure 18 B), reduced histopathological score (Figure 18 C), reduced neutrophil activity by measuring myeloperoxidase (MPO) activity (Figure 18 D) and robust inhibition of cytokine production (Figure 18 E) when compared to *Rassf1a*$^{+/-}$

mice treated with DSS. Indicating that inhibiting autophagy by using 3-MA protected RASSF1A knockout mice from DSS induce inflammation and 3-MA retained recovery for these mice from the aggressive inflammation caused by DSS.

5.2.3 Analysis of autophagic biomarker in colon lysates of 3-MA-treated mice

Using autophagy inhibitor 3-MA on RASSF1A knockout is the key to improving survival and recovery of this genotype from DSS induce inflammation. That means induce autophagy may be a factor on the poor survival for these genotypes. Using colon lysates of 3-MA-treated mice were blotted with p62. DSS-treated *Rassf1a*$^{+/-}$ mice with 3-MA revealed elevated levels of p62, indicating that autophagy inhibitor can decrease the autophagic activity in DSS-treated RASSF1A knockout mice (Figure 19 A-B). This result suggests that the loss of RASSF1A in *Rassf1a*$^{+/-}$ mice, lead to increase in the autophagic response under acute inflammation condition. As mentioned before, we speculate that under DSS treatment, *Rassf1a*$^{+/-}$ mice will lead to uncontrolled autophagic response via NOD2 that might be detrimental to epithelial repair and healing. All these data confirms that NOD2 has a critical role in inducing uncontrolled autophagy in RASSF1A knockout mice leading to induce inflammation and poor recovery. The use of 3-MA, autophagy inhibitor, improved the survival and reduce inflammation symptoms on these mice and that is due to 3-MA downregulation both autophagy and RIPK2 activation.

5.2.4 3-MA showed protection on *il10*$^{-/-}$ mice treated with DSS

Autophagy inhibitor, 3-MA protected DSS-treated *Rassf1a*$^{+/-}$ mice from damaging effects caused by the increased autophagic response therefore, we decided to explore the effectiveness of inhibiting autophagy using 3-MA in DSS-treated *Il10*$^{-/-}$ mice. *Il10*$^{-/-}$ mice were given 3% DSS in

drinking water for seven days followed by fresh water for recovery for another seven days during this time 3-MA was injected intraperitoneally on day 4 and 7. We speculated that inhibiting the autophagic response as early as day three might be detrimental to DSS-treated *il10*$^{-/-}$ mice as their autophagic response is regulated due to the presence of RASSF1A. Also, the clinical onset of the disease is delayed 1-2 days in DSS-treated *Il10*$^{-/-}$ mice when compared to DSS-treated *Rassf1a*$^{+/-}$ knockout mice. For these reasons, *Il10*$^{-/-}$ mice were treated with 3-MA on day 4 and day 6. *Il10*$^{-/-}$ mice are very sensitive to DSS, and they have to be euthanized between day seven and day ten due to massive rectal bleeding and increased disease indices. Autophagy inhibitor, 3-MA protected *Il10*$^{-/-}$ mice from DSS induced inflammation showing a significant increase in survival rate up to 60 % (Figure 20 A) and decrease in disease severity (Figure 20 B). Descending tissue sections showed active repair in the presence of 3-MA (Figure 20 C) reduced neutrophil activity (Figure 20 D) and robust inhibition of cytokine production (Figure 20 E) when compared to *Il10*$^{-/-}$ mice treated with DSS. Autophagy inhibitor 3-MA proved its effectiveness in *Il10*$^{-/-}$ mice in improving recovery when p62 expression level was measured in colon lysates for these genotypes (Figure 20 F). The data presented above, suggests that 3-MA reduced the damaging effects of DSS in *Il10*$^{-/-}$ mice by inhibiting the autophagic response that might be detrimental to epithelial repair and healing. More investigations have to be done to analyze the autophagic response in *Il10*$^{-/-}$ mice by exploring levels of p62 (a marker of autophy) and LC3-II (a marker of autophagosome formation) in colon lysates.

5.3 Conclusion:

Treatment with 3-MA resulted in low disease severity, reduced pro-inflammatory cytokines production, increased colon length, low histopathological scoring and increased epithelial cell proliferation in DSS-treated *Rassf1a*$^{+/-}$ mice. RASSF1A has a novel regulatory role

during the recovery phase post inflammation-induced injury by restricting the NOD2 induced autophagic response to inhibit inflammation and promote efficient epithelial repair. Inhibiting the autophagic response reversed the damaging effects of DSS inflammation injury. *Il10*$^{-/-}$ mice and showed a significant increase in survival rate, decrease in disease severity, decrease in pro-inflammatory cytokines production and increase in epithelial cells proliferation upon DSS administration. Based on data presented in this chapter we suggest that the DSS damage in *Il-10*$^{-/-}$ mice might be due to active autophagic response, indicated that DSS treated *Il10* $^{-/-}$ mice were protected from induced inflammation when the autophagic response is inhibited by 3-MA. DSS treated *Il10*$^{-/-}$ mouse is an interesting mouse model for IBD drug study and pharmacological inhibition of the autophagic response might be a novel therapeutic approach for IBD treatment and so preventing patients from predisposing to cancer later in life.

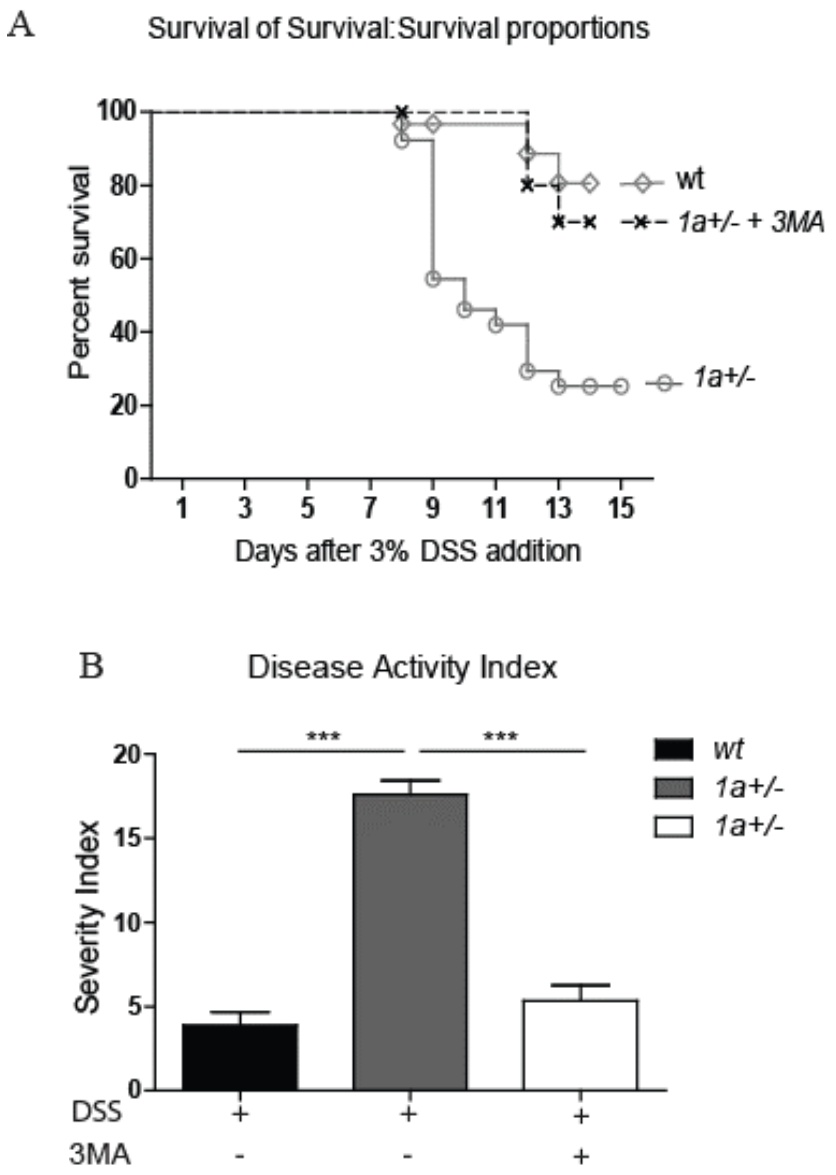


Figure 17: Autophagy inhibitor 3-MA showed improve survival of *Rassf1a-/-* upon DSS administration. (A) A Kaplan-Meier curve is monitoring % survival following DSS treatment. n = 23-28. p value for DSS-treated *Rassf1a*$^{+/-}$ mice (- /+ 3-MA) = 0.056 (B) Disease activity index (DAI) following DSS treatment. P value <0.0001 (***), n = 6-13 (Data courtesy of Yahya Fiteih).

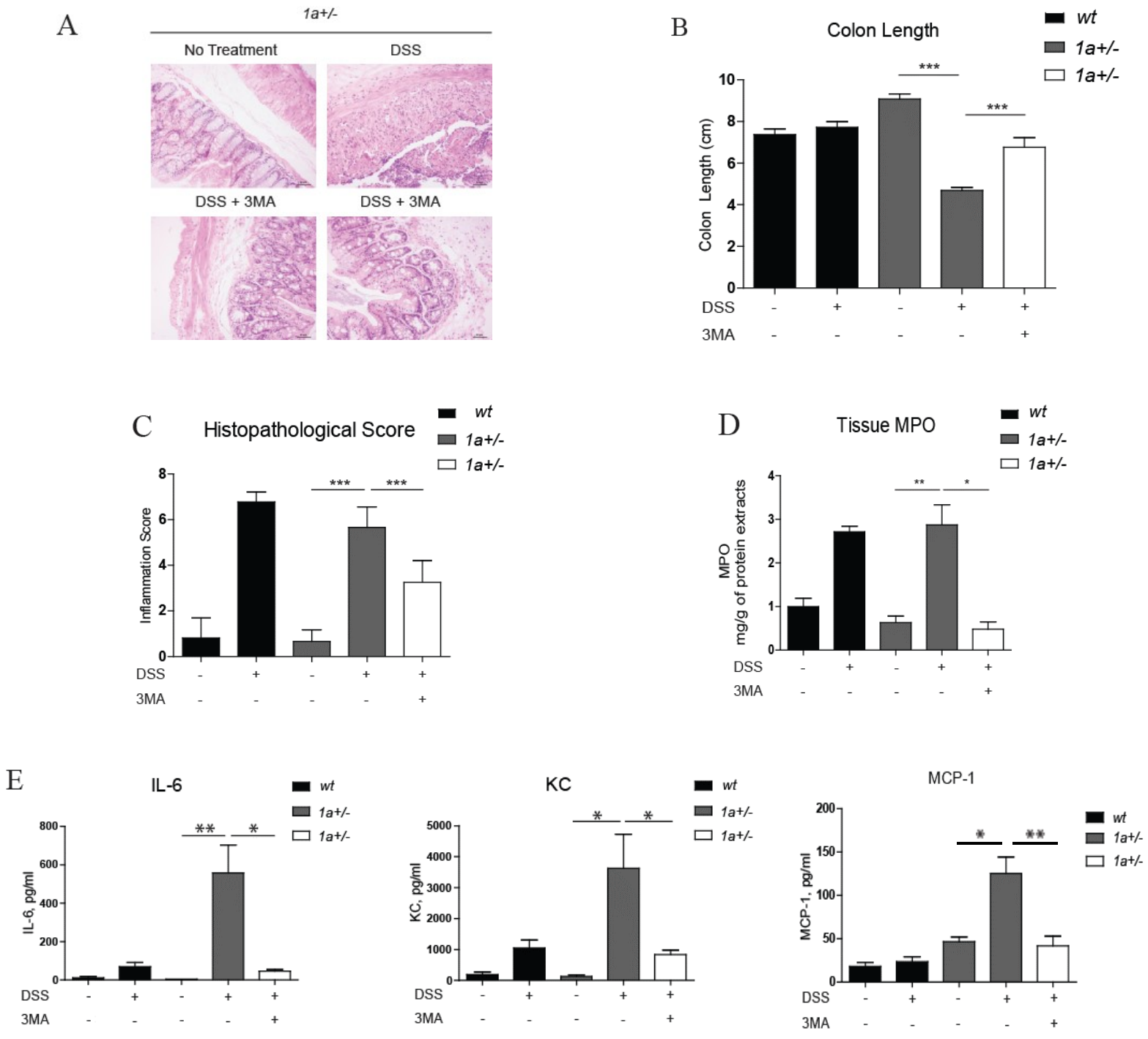


Figure 18: 3-MA protected *Rassf1a*$^{-/-}$ from DSS induced inflammation. (A) The longitudinal cross-section of the descending colon stained with H&E. (Data courtesy of Yahya Fiteih). (B) Colon length for treated mice was measured. P value <0.0001 (***). n=10-20. (C) A pathologist blindly measured inflammation scoring of colon sections. P value are <0.0001 and 0.0004 (***). n= 6-13. (Data courtesy of Yahya Fiteih) (D) Tissue MPO activity as determined by ELISA. P value are

0.0034 (**) and 0.0103 (*). n = 4-11. (E) Blood serum of the treated animals was analyzed for cytokine production using color-coded polystyrene beads technology. P value for *Il-6* are 0.0020 (**) and 0.0246 (*). n=3-5. P value for KC are 0.0137 and 0.0375 (*). n=5. P value for MCP-1 are 0.0161 (*) and 0.0050 (**) n=4-6.

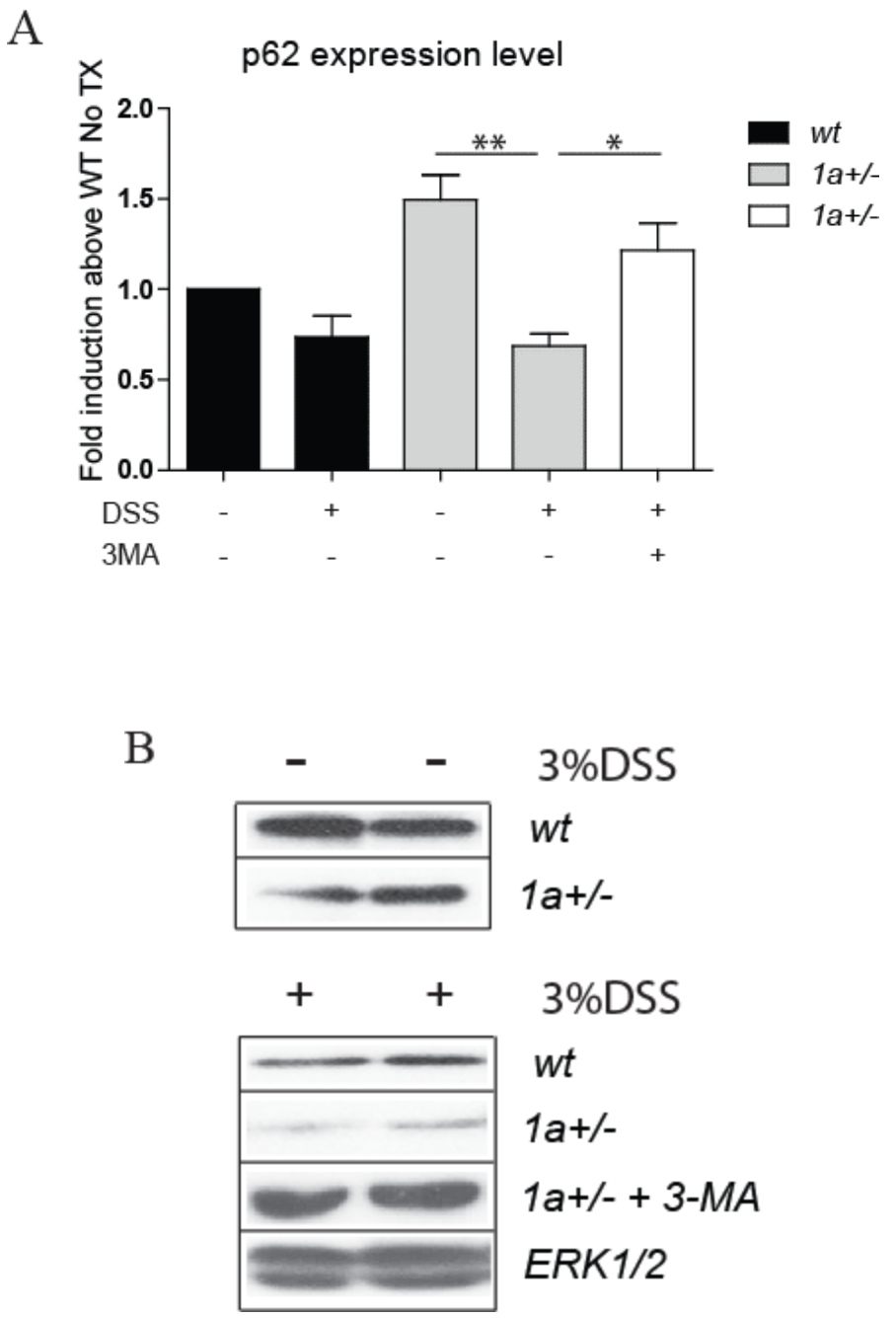
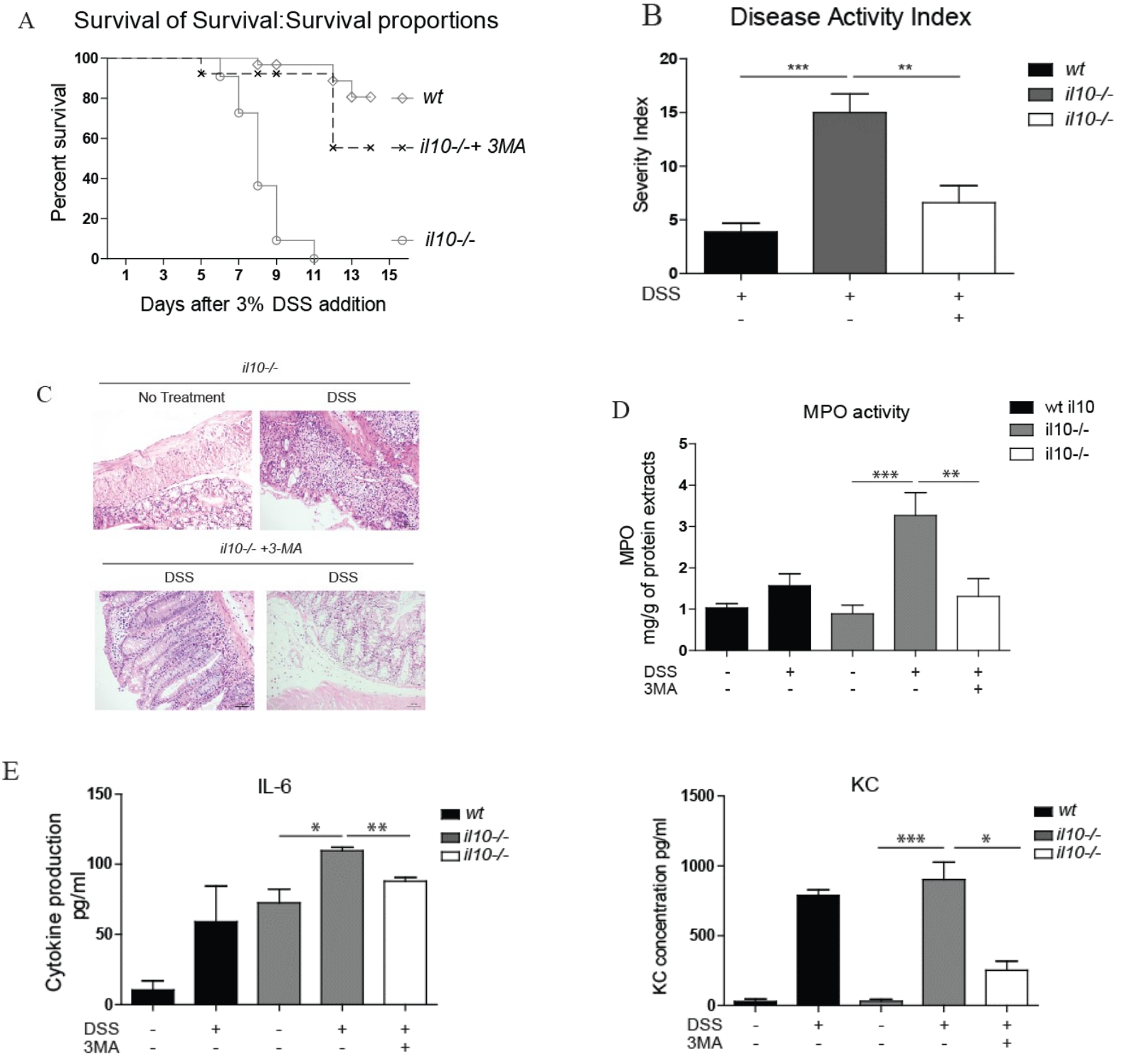


Figure 19: Analysis of autophagic biomarker in colon lysates of 3-MA-treated mice. (A) Colon lysates from treated mice blotted against p62 and expression level was quantified and normalized to wild type. P value 0.0037 (**), 0.0139 (*). n= 3-5. (B) Analysis for p62 expression level following *in vivo* DSS insult in colon lysate.


A
Survival of Survival:Survival proportions
Percent survival
Days after 3% DSS addition
wt
il10-/-+ 3MA
il10-/-
B
Disease Activity Index
Severity Index
DSS
wt
il10-/-
il10-/-
C
il10-/-
No Treatment
DSS
il10-/- +3-MA
DSS
DSS
D
MPO activity
MPO
mg/g of protein extracts
DSS
3MA
wt il10
il10-/-
il10-/-
E
IL-6
Cytokine production
pg/ml
DSS
3MA
wt
il10-/-
il10-/-
KC
KC concentration pg/ml
DSS
3MA
wt
il10-/-
il10-/-

F

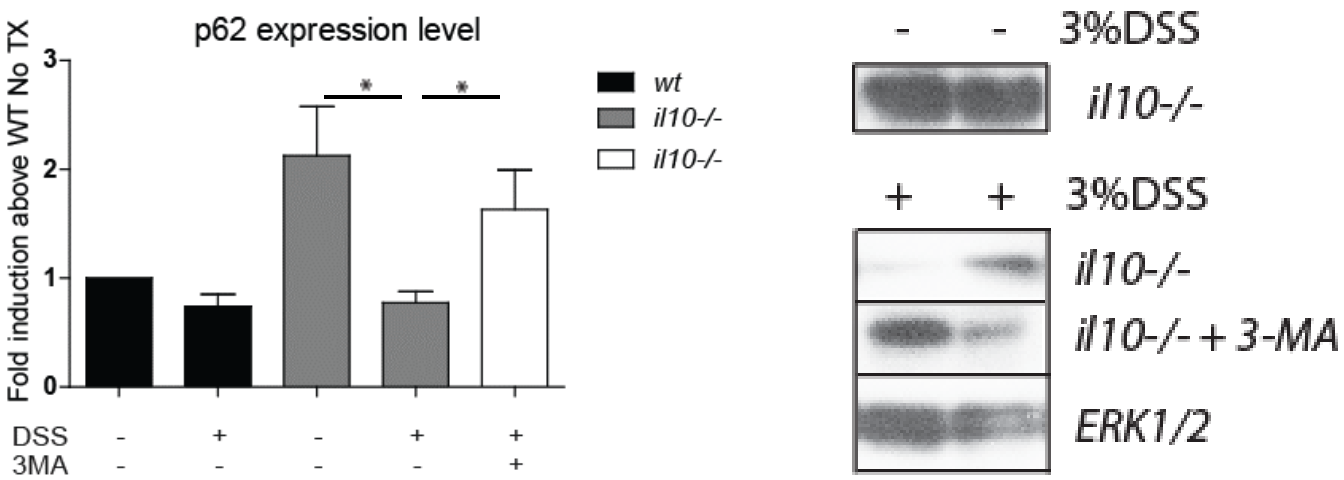


Figure 20: 3-MA showed protection on *Il10*$^{-/-}$ mice treated with DSS. (A) A Kaplan-Meier curve is monitoring % survival following DSS treatment. n= 11-28. p value for DSS-treated *Il10*$^{-/-}$ mice (- /+ 3-MA) = 0.049 (Data courtesy of Yahya Fiteih) (B) Disease activity index (DAI) of these mice were monitored following DSS treatment. P value are <0.0001 (***) and 0.0017 (**). n= 11-28. (Data courtesy of Yahya Fiteih). (C) A representative picture of the colon from the indicated genotypes treatments is shown. (Data Courtesy of Yahya Fiteih) (D) Tissue MPO activity as determined by ELISA. P value are 0.0039 (**) and 0.0238 (*). n= 4-5. (E) Blood serum of the treated animals was analyzed for cytokine production using color-coded polystyrene beads technology. P value 0.0214 (*) and 0.0019 (**). n= 3-5. (F) Colon lysates from treated mice blotted against p62 and expression level was quantified and normalized to wild type. P value are 0.0412 (*) and 0.0215 (*) n= 3-5.

6 Chapter Six-Inhibition of RIPK2 downstream signaling pathway of NOD2 showed protection against inflammation.

6.1 Rationale:

All the results so far suggested the importance of an active NOD2 signaling pathway in driving DSS-induced inflammation injury in *Rassf1a*$^{-/-}$ mice. For NOD2 activation, RIPK2 activation is required to promote both NFκB activation and an autophagic response. Genetic disruption of the *Nod2/Ripk2* in mice, showed dysbiotic intestinal flora leading to intestinal inflammation [(210)] and the loss of RIPK2 resulted to the inability of cells to carry out mitophagy leading to enhanced production of superoxide/reactive oxygen species by mitochondria and accumulation of damaged mitochondria that will trigger a capase-11 dependent inflammasome activation.[(211, 212)] For such reasons inhibiting RIPK2 may consider an alternate novel therapeutics for the treatment of abnormal inflammation and autophagic states that is driven by the NOD2/RIPK2 pathway.

Most RIPK2 inhibitors do not to inhibit RIPK2 as their primary target. Thus, inhibition of RIPK2 is an off-target effect of these drugs such as Gefitinib and Regorafenib [(213)]. Another RIPK2 inhibitor was isolated by GlaxoSmithKline which is GSK-583. GSK-583 found to bind to the ATP binding pocket of the kinase domain and inhibit RIKP2 with an IC50 of (5 – 50 nM) [(214)]. Recently, two other RIPK2 inhibitors were identification to inhibit 92% of RIPK2 activity at 100 nM and they were also able to inhibit alleviate inflammation in a rodent model for inflammatory bowel disease these inhibitors are OD36 and OD38 [(215)]. However, like most RIPK2 inhibitors these molecules showed off-target inhibition of Fyn, TGFB2, ALK-2, and Lck. Using molecular modeling and cheminformatics analyses, in collaboration with Dr. Carlos Velazquez Martinez, two RIPK2 inhibitor molecules were identified utilizing c-ABL kinase inhibitor, ponatinib. That was done by first looking at the crystal structure of RIPK2 and its association with ponatinib. Then searching for structural matches to ponatinib that have lower free energies and more efficient in

inhibiting RIPK2 that was through the chemical database. Also, two more layers were added in the search which was compounds that inhibited EGFR > 1000 nM and that had higher free energies for docking with EGFR and c-ABL. Nothing appears to be known till now about these compounds, but since RIPK2 is the downstream signaling pathway of NOD2 which is linked to NFκB and autophagy activation, we speculate that the *in vivo* use of our selective RIPK2 inhibitors will target this NOD2/RIPK2 non-canonical activation pathway for NFκB.

6.2 Results:

6.2.1 RIPK2 activity was highly elevated in *Rassf1a*$^{-/-}$ mice treated with DSS and in IBD patients

Upon NOD2 activation RIPK2 gets recruited and activated. Upon activation, RIPK2 gets autophosphorylates in two different sites Ser176 and on Tyr474. Serine 176 phosphorylation is essential for RIPK2 activity and tyrosine 474 phosphorylation as necessary for effective NOD2 signaling [(216, 217)]. Our objective in this chapter is to monitor RIPK2 activity in RASSF1A treated mice upon DSS administration. To test that our lab has designed a proprietary antibody used for detecting phosphorylation of RIPK2 at tyrosine 474 site. The antibody specificity was tested *in vitro* and *in vivo*. *In vivo* the antibody was tested by immunohistochemistry on colon sections harvested from *Rassf1a*$^{-/-}$ (Figure 21 A) and *Il10*$^{-/-}$ (Figure 21 B) upon DSS administration. We were able to detect elevated tyrosine phosphorylated RIPK2 in DSS-treated *Rassf1a*$^{-/-}$ and *Il10*$^{-/-}$ mice suggesting hyperactivation of the NOD2/RIPK2 pathway in the ulcerative colitis model. We started to investigate RIPK2 activity on colon sections harvested from *Rassf1a*$^{-/-}$ mice treated with 3-MA using immunohistochemistry. We observed a reduction in RIPK2 activity upon treating *Rassf1a*$^{-/-}$ mice with autophagy inhibitor 3-MA. These results support our hypothesis that the poor survival and induced inflammation caused by DSS in *Rassf1a*$^{-/-}$ is due to NOD2/RIPK2

uncontrolled activation and RASSF1A is important in regulating NOD2/RIPK2 signaling pathway. Our observation also supports that autophagy inhibitor 3-MA may consider a therapeutic novel to downregulate NOD2/RIPK2 activation. We then wanted to monitor RIPK2 activity in colon snips taken from IBD patients. The colon snips from IBD patients were tested against phospho-Y474 RIPK2 antibody by immunohistochemistry. We observed that both CD and UC showed induce the activity of RIPK2 when compared to colon snips take from control patients (Figure 21 C). Indicating that targeting RIPK2 activity will be a novel therapeutic approach to treat IBD and for such reason RIPK2 Inhibitor-1 was synthesized.

6.2.2 RIPK2 inhibitor showed improve survival for *Rassf1a*$^{-/-}$ mice upon DSS administration

Upon administrating *Rassf1a*$^{-/-}$ mice with DSS, we realized an induction of RIPK2 activity, and for such reason, we were interested in seeing the outcome of inhibiting RIPK2 in *Rassf1a*$^{-/-}$ colitis model. As previously mentioned with collaboration with Dr. Carlos Velazquez Martinez, several RIPK2 inhibitors hits were identified. One of these inhibitors which are RIPK2 Inhibitor-1 was tested in *Rassf1a*$^{-/-}$ mice upon DSS administration. *Rassf1a*$^{+/-}$ mice (10-14 weeks old) were given 3% DSS in drinking water for seven days followed by fresh water for recovery for another seven days. Different concentrations of RIPK2 inhibitor-1 were tested and the concentration that showed significant outcome was 1 μg/g body weight. RIPK2 inhibitor-1 (1 μg/g body weight) was administrated by intraperitoneal injections on day 3 and 6. Interestingly, RIPK2 inhibitor-1 significantly increased the survival rate of DSS-treated *Rassf1a*$^{+/-}$ mice to >70% when compared with *Rassf1a*$^{+/-}$ mice treated only with DSS which have survival rate about 25% (Figure 22 A). Also, there was a significant decrease in disease severity for DSS-treated *Rassf1a*$^{+/-}$ mice upon using RIPK2 inhibitor-1 (Figure 22 B). This indicates that RIPK2 was an important driver of

inflammation injury in DSS-treated *Rassf1a*$^{-/-}$ mice and by inhibiting RIPK2 activity survival and disease activity was recovered in *Rassf1a*$^{-/-}$ mice.

6.2.3 Mice treated with RIPK2 inhibitor showed reduced inflammation

Furthermore, to investigate the inflammation outcome of using RIPK2 inhibitor-1on DSS treated mice. The mice were administrated with DSS for seven days and in day seven the DSS was replaced with water for one day for recovery during the seven days the mice were treated with RIPK2 inhibitor-1 in day three and six by intraperitoneal injections. On day eight the mice were euthanized and both blood and colon sections were harvested for these mice for inflammation analysis. Reduction in histopathological score was detected in *Rassf1a*$^{-/-}$ upon treating them with RIPK2 inhibitor-1 when compared with *Rassf1a*$^{-/-}$ (Figure 23 A). Significantly, RIPK2 inhibitor-1 treated mice showed low cytokine production an indication of low NFκB activity (Figure 23 B). We further proved the role of RIPK2 in regulating NFκB in vitro by measuring NFκB activity in HCT 116 upon MDP stimulation and we found that RIPK2 inhibitors down-regulated NFκB activity (Figure 23 C-D).

6.3 Conclusion:

The above data support the outcome of inhibiting RIPK2 activity on inflammation. RIPK2 activation is highly regulated in *Rassf1a*$^{-/-}$ and *Il10*$^{-/-}$ mice upon DSS administration, and upon using 3-MA inhibitor, we observed a down-regulation of RIPK2 activity in these genotypes. Form the previous observation RIPK2 Inhibitor-1 was synthesized and administrated in *Rassf1a*$^{-/-}$ mice. Upon inhibiting RIPK2 activity, *Rassf1a*$^{-/-}$ mice showed improve survival and recovery from induced inflammation symptoms caused by uncontrolled activation of the NOD2 signaling pathway. Further investigation was done on the inflammation come upon using this inhibitor, and

we observed a reduction in cytokine production. Histopathological scoring was done on colon sections isolated from mice treated with RIPK2 Inhibitor-1 and the results revealed a reduction in disease severity. Indicating signs of recovery in these mice after administrating them with the inhibitor. We further tested the RIPK2 Inhibitor-1 effect on NFκB *in vitro.* Using NFκB reporter assay and EMSA the results showed a reduction in NFκB activity when RIPK2 Inhibitor-1 was used. Indicating that RIPK2 may be considered another therapeutic target to treat induced inflammation in IBD patient and it may consider an option to avoid cancer development in IBD patients later in life.

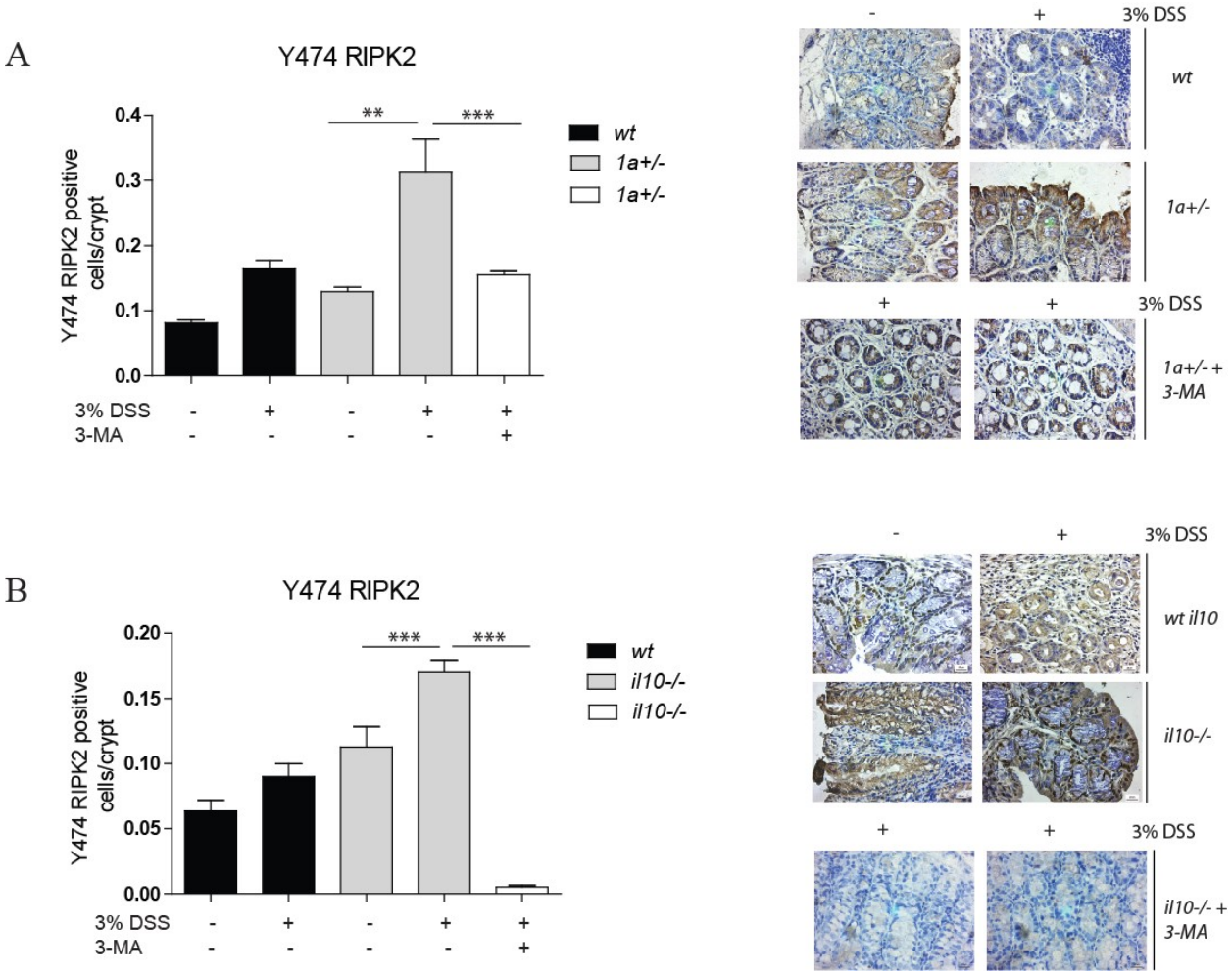


Figure 21: RIPK2 activity was highly elevated in Rassf1a$^{-/-}$ mice treated with DSS and in IBD patients. (A) Analysis of pY474-RIPK2 expression by IHC was carried out as indicated on colonic tissue sections on day nine post-DSS treatment of *Rassf1a*$^{-/-}$ and *Rassf1a*$^{-/-}$ treated with 3MA (right panel) and expression of pY474 RIPK2 in colonic tissue from day 9 DSS-treated animals as indicated (fold induction are graphed in the left panel). P value 0.0015 (**) and <0.0001 (***). n= 4-16. (B) Analysis of pY474-RIPK2 expression by IHC was carried out as indicated on colonic tissue sections on day nine post-DSS treatment of *Il10*$^{-/-}$ and *Il10*$^{-/-}$ treated with 3MA (right panel) and expression of pY474 RIPK2 in colon lysates from day 9 DSS-treated animals as indicated (fold induction are graphed in the left panel). P value <0.0001 (***). n= 12-16.

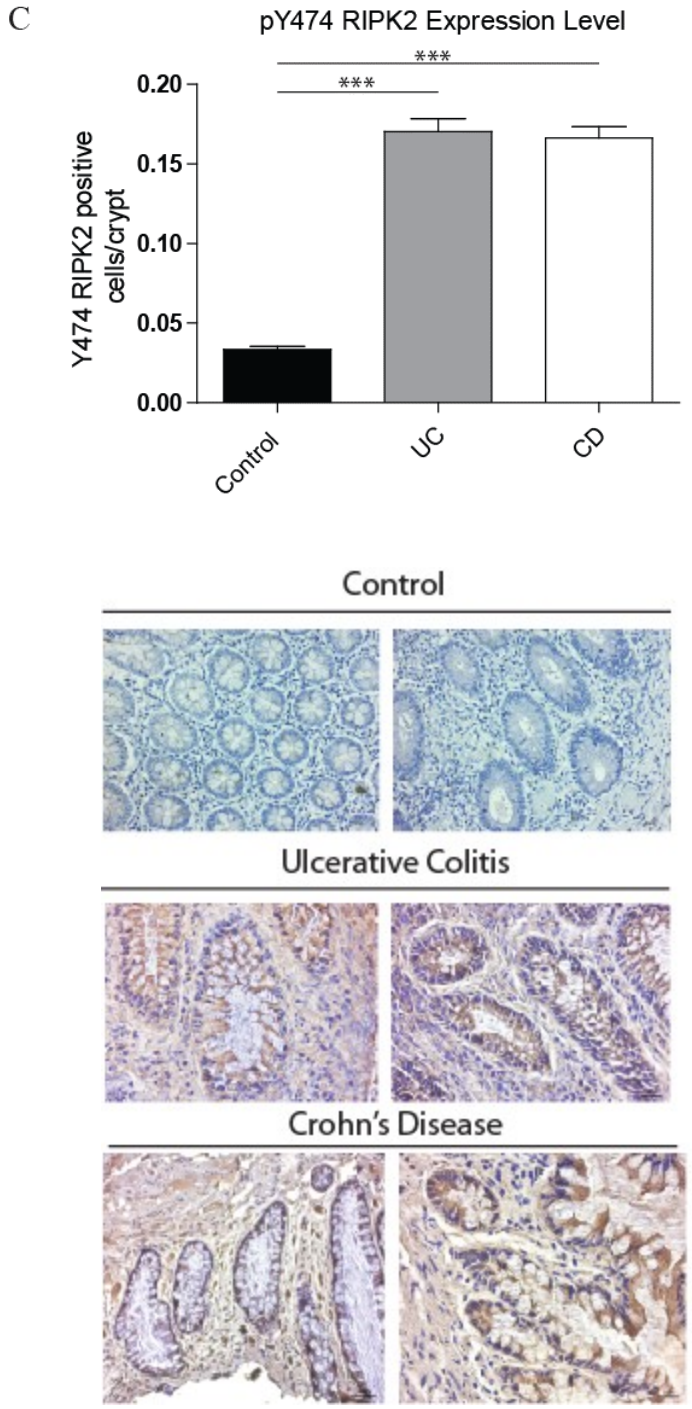


Figure 21: RIPK2 activity was highly elevated in IBD patients. (C) Analysis of active RIPK2 in patient's tissue was performed via immunohistochemistry analysis using the pY474 RIPK2 antibody. The top panel is 1000 cells scored for the nuclear presence of RIPK2 Y474, and the bottom panel is a figure of the IHC using pY474 RIPK2 antibody. P value is <0.0001 (***). n = 5-15.

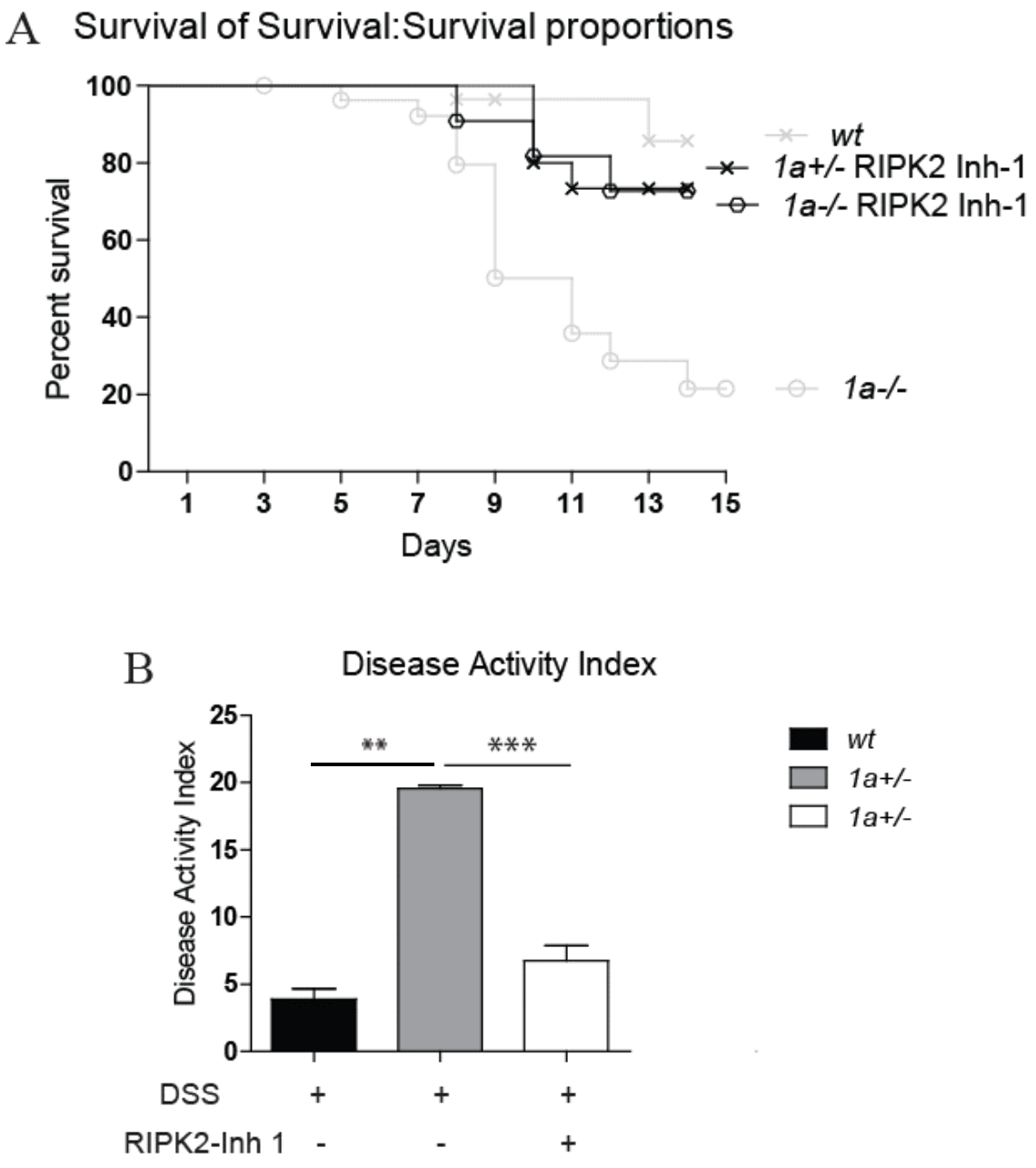


Figure 22: RIPK2 inhibitor showed improve survival for *Rassf1a*$^{-/-}$ mice upon DSS administration. (A) A Kaplan-Meier curve monitoring % survival following DSS treatment. p value for DSS-treated *1a*$^{-/-}$ mice (-/+ RIPK2 inhibitor 1) = P<0.0001 n = 18-28. (B) Disease activity index (DAI) following DSS treatment. P value <0.0001 (***) and 0.0083 (**), n = 6-13.

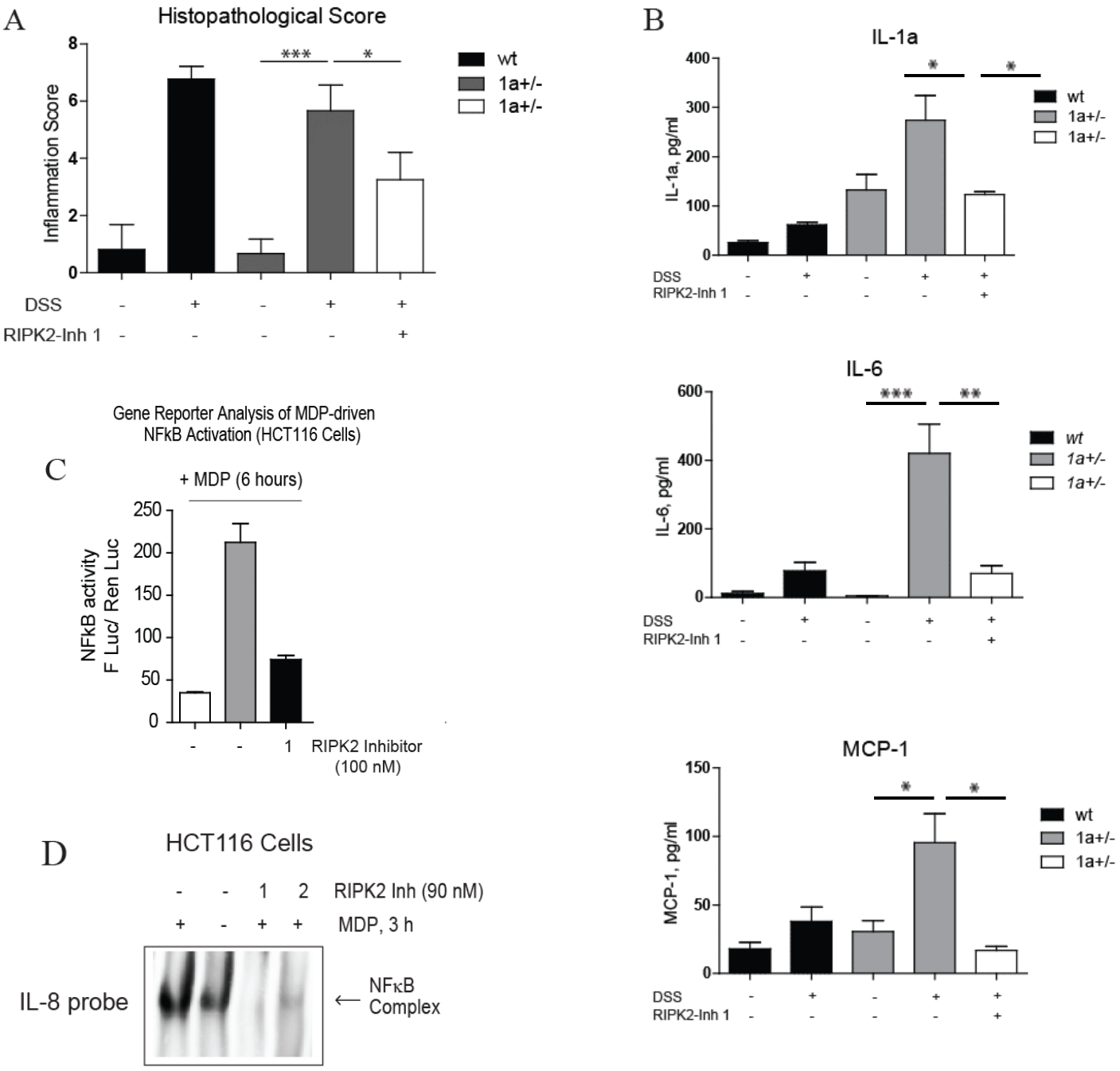


Figure 23: Mice treated with RIPK2 inhibitor showed reduced inflammation. (A) A pathologist blindly measured inflammation scoring of colon sections. P value <0.0001 (***) and 0.0196 (*), n = 5-13. (B) Blood serum of the treated animals was analyzed for cytokine production using color-coded polystyrene beads technology. P value for IL-1a are 0.0448 (*) and 0.0244 (*). P value for

IL-6 are P<0.0001 (***) and 0.0019 (**). P value for MCP-1 are 0.0135 and 0.0374 (*). n=3-5. (C) NFκB gene reporter assay determination of inhibition of MDP stimulated NFκB activity with RIPK2 inhibitors. (Data courtesy of Mohammed Salla). (D) EMSA was carried out using the NFκB binding site on the IL-8 promoter. HCT116 cells were pre-treated with the indicated RIPK2 inhibitors for two days, followed by stimulation with MDP and nuclear/cytoplasmic extraction. (Data and figure for panel D provided by Dr. Shairaz Baksh).

7 Chapter Seven-Discussion and Future Experiments

7.1 Discussion:

NOD2 and ATG16L1 are considered among the susceptible genes associated with IBD. Polymorphisms of NOD2 were detected in CD patients and with childhood onset of the CD. NOD2 is a pathogen recognition receptor that plays a critical role in regulating immune response upon pathogen invasion by inducing pre-inflammatory cytokine production and bacterial clearance. ATG16L1 is an important gene in the autophagy signalling pathway and this gene is associated with NOD2 for bacterial clearance. Polymorphisms of ATG16L1 in IBD patients was detected, and hematopoietic cells from *Atg16*$^{-/-}$ mice showed induction of inflammatory cytokines, IL-1β and IL-18 levels [192, 197]. Indicating that both NOD2 and ATG16L1 are important for regulating intestinal immune defence and tolerance [91]. RASSF1A is a tumor suppressor gene that was found to be associated with IBD and acts as a trigger for autophagy to suppress genome instability through MAP1S/C19ORF5 interacting protein [101, 218]. Since RASSF1A and NOD2 genes are associated with IBD understanding the correlation between these two genes was our hypothesis and to test our hypothesis we used *Rassf1a*$^{-/-}$ colitis model. Knockout mice have helped in understanding the pathophysiological mechanism responsible for IBD initiation and progression, but unfortunately, IBD etiology remains completely unknown. The *Rassf1a*$^{-/-}$ mice model is useful because we have observed extreme sensitivity of these mice to DSS induced inflammation injury, RASSF1A epigenetically silenced during inflammation induced injury and in IBD patients (Salla et al., unpublished observations) and evidence that RASSF1A can associate with several elements linked to NFκB, such as TLRs [80], NOD2 and TBK1 (Salla et al., unpublished observations). Indicating that RASSF1A knockout mice are a useful model for studying the molecular mechanism of IBD colitis.

Autophagic regulation was detected in colon lysates from *Rassf1a*$^{-/-}$ mice upon DSS administration. Indicating that RASSF1A might have a role in regulating autophagy in these mice and its absences caused induce autophagy. Our question was does RASSF1A regulate autophagy through NOD2 and we tested that by using *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$. *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ mice showed improve survival and recovery upon DSS administration suggesting that NOD2 is the driver gene for inflammation in *Rassf1a*$^{-/-}$ mice. We then wanted to test if RASSF1A does regulates NOD2 signalling pathway. *In vitro* studies showed that RASSF1A can physically associates with NOD2 upon MDP stimulation and such association interfered RIPK2 and ATG16L1 association with NOD2 leading to NOD2 downregulation. Indicating that the loss RASSF1A resulted in uncontrolled autophagic response and inflammation via NOD2 and RASSF1A is essential in controlling NOD2 induce activation.

We then wanted to improve the survival and reduce the inflammation outcome of *Rassf1a*$^{-/-}$ under the caused by DSS. Since autophagy was found to be induced, 3-MA, autophagy inhibitor, was injected in these mice and the outcome of using 3-MA was improve survival and reduce inflammation. p62, autophagy biomarker, was tested in the colon lysates of 3-MA treated mice and we observed accumulation of p62 expression level. These results indicate the importance of RASSFA in regulating autophagy through NOD2. Another experiment was done to confirm that NOD2 is responsible for triggering inflammation in these genotypes. That experiment was analysing RIPK2 activation in the colon lysates of 3-MA treated mice. Surprisingly, 3-MA treated mice showed reduction in RIPK2 activity when compared to *Rassf1a*$^{-/-}$ treated with DSS only. Since we had promising results with 3-MA in *Rassf1a*$^{+/-}$ mice treated with DSS, we decided to test our hypothesis on an established IBD mouse model like *Il10*$^{-/-}$. Interestingly, using 3-MA in *Il10*$^{-/-}$ mice treated with DSS revealed a reduction in the damaging effects caused by DSS. Indicating

that 3-MA may consider a therapeutic choice to threated induce inflammation caused by NOD2 induce activation.

Another inhibitor was tested on *Rassf1a*$^{-/-}$ mice and that was the RIIPK2 Inhibitor-1. RIPK2 is an adopter protein that binds to NOD2 upon MDP sensing to regulate autophagy and inflammation. Not only RIPK2 activity was downregulated in *Rassf1a*$^{-/-}$ and *Il10*$^{-/-}$ mice treated with 3-MA but also induce RIPK2 activity was observed in biopsy samples from IBD patients when compared to control. Indicating the importance of targeting RIPK2 activity to downregulate inflammation in *Rassf1a*$^{-/-}$. Using RIPK2 inhibitor-1, RASSF1A knockout mice showed promising results by inducing the survival rate and improving recovery. RIPK2 inhibitor-1 proved to inhibit RIPK2 induce activation and its signaling pathway. That was proved by monitoring the both RIPK2 and NFκB activity, and it was realized that upon using RIPK2 inhibitor-1 both RIPK2 and NFκB activity was reduced. Indicating that RIPK2 Inhbitior-1 may consider another therapeutic choice to treat inflammation caused by NOD2 induce activation. From all these data, this thesis project suggests the importance of RASSF1A in regulating NOD2 signalling pathway. It also shows that NOD2 is a trigger gene for inflammation in colitis model suggesting the role of NOD2 in UC pathogenesis. Finally this thesis proves that either 3-MA or RIPK2 inhibitor-1 could be novel therapeutic approaches to enhance recovery from inflammation-induced injury and be useful in treating and saving IBD patients from increased risk of getting cancer later in life.

7.2 Future Directions:

Based on results from this thesis, future experiments will include: (1) Detailed analysis of the molecular association of RASSF1A and NOD2. This can be accomplished by exploring the interaction between different constructs of NOD2 with RASSF1A by immunoprecipitation (I.P) experiments, utilizing NOD2 deletions within the N terminal CARD domain, C-terminal leucine-rich repeat and nucleotide binding domain will also be used [(217)]. Understanding RASSF1A/NOD2 association may allow locking NOD2 in an inactive conformation and inhibit downstream activation of autophagy and/or NF-κB activation by using a small molecule design of peptide sequences to match the interacting surface of RASSF1A/NOD2. (2) Investigating the association of RASSF1A with NOD1 upon DAP stimulation. Overexpressing RASSF1A in HCT116 cells and immunoprecipitation against NOD1 upon DAP. NOD1 and NOD2 are members of the NLRs, different peptidoglycans activate them but what they both activate RIPK2. Depending on which domain is important for RASSF1A association with NOD2, RASSF1A might also associate with NOD1 regulating inflammation and autophagy caused by NOD2. (3) Another future experiment is to investigate autophagy regulation in 3-MA, and RIPK2-Inhibitor treated mice. Two possible biomarkers can be used to monitor autophagy regulation which is LC3 and Beclin 1. ATG12, ATG5 and ATG16L1 complex promotes LC3 lipidation and assists in the insertion of the LC3-II, the modified form of LC3-I, to the phagophore. Monitoring LC3 modification from LC3-I to LC3-II can be tested in the colon lysates of these treated mice by immunoblotting. The second biomarker for autophagy regulation is beclin-1. Beclin1 (BECN1) induces autophagy through forming class III PI3K complex (PIK3C3). PIK3C3 interacts with autophagy related 14 (ATG14) which is important for recruitment of ATG16L1 complex and LC3/ATG8, to the autophagosome [(219)]. Beclin-1 (BECN1) is an essential protein in the autophagy signaling pathway. Autophagy is

induced by the release of BECN1 from B-cell lymphoma 2 (BCL2) by pro-apoptotic BH3 proteins through phosphorylation of BECN1 by death-associated protein kinase 1 (DAPK1) at Thr119 [(220)]. Immunohistochemistry using phospho-Thr119 in BECN1 (EMD millipore, cat# ABC118) can be done on colon of treated mice to analyze beclin regulation. The aim of these two experiments to study autophagy regulation in 3-MA and RIPK2 Inhibitor-1 treated mice since we showed regulation of p62 expression level in these mice. (5) Another future experiment to study autophagy regulation *in vitro* in the presences of RASSF1A. Explore the role of RASSF1A in regulating Beclin-1 complex upon nutrient deprivation. HCT116 overexpressed with RASSF1A-HA will be immunoprecipitated with total BCL-2 (Santa Cruz, cat# sc-509) and blotted against phospho-Thr119 in BECN1 (EMD millipore, cat# ABC118). This experiment aims to confirm the role of RASSF1A in regulating autophagy in the presences of NOD2.

Information obtained from these future experiments will firmly ascertain the importance of RASSF1A in regulating autophagy through NOD2. Furthermore, information obtained from my thesis will assist in understanding the importance of RASSF1A in modulating inflammatory signaling and its role a negative regulator of NFκB.

Reference:

1. Baumgart, D. C., and Sandborn, W. J. (2007) Inflammatory bowel disease: clinical aspects and established and evolving therapies, *Lancet 369*, 1641-1657.
2. Khor, B., Gardet, A., and Xavier, R. J. (2011) Genetics and pathogenesis of inflammatory bowel disease, *Nature 474*, 307-317.
3. Loftus, E. V., Jr. (2004) Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences, *Gastroenterology 126*, 1504-1517.
4. M'Koma, A. E. (2013) Inflammatory bowel disease: an expanding global health problem, *Clinical medicine insights. Gastroenterology 6*, 33-47.
5. Bernstein, C. N., Wajda, A., Svenson, L. W., MacKenzie, A., Koehoorn, M., Jackson, M., Fedorak, R., Israel, D., and Blanchard, J. F. (2006) The epidemiology of inflammatory bowel disease in Canada: a population-based study, *The American journal of gastroenterology 101*, 1559-1568.
6. Kaplan, G. G. (2015) The global burden of IBD: from 2015 to 2025, *Nature reviews. Gastroenterology & hepatology 12*, 720-727.
7. Benchimol, E. I., Guttmann, A., Griffiths, A. M., Rabeneck, L., Mack, D. R., Brill, H., Howard, J., Guan, J., and To, T. (2009) Increasing incidence of paediatric inflammatory bowel disease in Ontario, Canada: evidence from health administrative data, *Gut 58*, 1490-1497.
8. Podolsky, D. K. (2002) Inflammatory bowel disease, *The New England journal of medicine 347*, 417-429.
9. Kugathasan, S., and Fiocchi, C. (2007) Progress in basic inflammatory bowel disease research, *Seminars in pediatric surgery 16*, 146-153.
10. Thomas, G. A., Rhodes, J., Green, J. T., and Richardson, C. (2000) Role of smoking in inflammatory bowel disease: implications for therapy, *Postgraduate medical journal 76*, 273-279.
11. Birrenbach, T., and Bocker, U. (2004) Inflammatory bowel disease and smoking: a review of epidemiology, pathophysiology, and therapeutic implications, *Inflammatory bowel diseases 10*, 848-859.

12. Garg, M., Lubel, J. S., Sparrow, M. P., Holt, S. G., and Gibson, P. R. (2012) Review article: vitamin D and inflammatory bowel disease--established concepts and future directions, *Alimentary pharmacology & therapeutics 36*, 324-344.
13. Leslie, W. D., Miller, N., Rogala, L., and Bernstein, C. N. (2008) Vitamin D status and bone density in recently diagnosed inflammatory bowel disease: the Manitoba IBD Cohort Study, *The American journal of gastroenterology 103*, 1451-1459.
14. Ooi, J. H., Li, Y., Rogers, C. J., and Cantorna, M. T. (2013) Vitamin D regulates the gut microbiome and protects mice from dextran sodium sulfate-induced colitis, *The Journal of nutrition 143*, 1679-1686.
15. Ananthakrishnan, A. N., Higuchi, L. M., Huang, E. S., Khalili, H., Richter, J. M., Fuchs, C. S., and Chan, A. T. (2012) Aspirin, nonsteroidal anti-inflammatory drug use, and risk for Crohn disease and ulcerative colitis: a cohort study, *Annals of internal medicine 156*, 350-359.
16. Shaw, S. Y., Blanchard, J. F., and Bernstein, C. N. (2010) Association between the use of antibiotics in the first year of life and pediatric inflammatory bowel disease, *The American journal of gastroenterology 105*, 2687-2692.
17. Mawdsley, J. E., and Rampton, D. S. (2006) The role of psychological stress in inflammatory bowel disease, *Neuroimmunomodulation 13*, 327-336.
18. Camara, R. J., Schoepfer, A. M., Pittet, V., Begre, S., von Kanel, R., and Swiss Inflammatory Bowel Disease Cohort Study, G. (2011) Mood and nonmood components of perceived stress and exacerbation of Crohn's disease, *Inflammatory bowel diseases 17*, 2358-2365.
19. Thia, K. T., Loftus, E. V., Jr., Sandborn, W. J., and Yang, S. K. (2008) An update on the epidemiology of inflammatory bowel disease in Asia, *The American journal of gastroenterology 103*, 3167-3182.
20. Kaplan, G. G., Hubbard, J., Korzenik, J., Sands, B. E., Panaccione, R., Ghosh, S., Wheeler, A. J., and Villeneuve, P. J. (2010) The inflammatory bowel diseases and ambient air pollution: a novel association, *The American journal of gastroenterology 105*, 2412-2419.
21. Ananthakrishnan, A. N., McGinley, E. L., Binion, D. G., and Saeian, K. (2011) Ambient air pollution correlates with hospitalizations for inflammatory bowel disease: an ecologic analysis, *Inflammatory bowel diseases 17*, 1138-1145.

22. Opstelten, J. L., Beelen, R. M. J., Leenders, M., Hoek, G., Brunekreef, B., van Schaik, F. D. M., Siersema, P. D., Eriksen, K. T., Raaschou-Nielsen, O., Tjonneland, A., Overvad, K., Boutron-Ruault, M. C., Carbonnel, F., de Hoogh, K., Key, T. J., Luben, R., Chan, S. S. M., Hart, A. R., Bueno-de-Mesquita, H. B., and Oldenburg, B. (2016) Exposure to Ambient Air Pollution and the Risk of Inflammatory Bowel Disease: A European Nested Case-Control Study, *Digestive diseases and sciences 61*, 2963-2971.

23. Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J. M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H. B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Dore, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Meta, H. I. T. C., Bork, P., Ehrlich, S. D., and Wang, J. (2010) A human gut microbial gene catalogue established by metagenomic sequencing, *Nature 464*, 59-65.

24. Ahmed, I., Roy, B. C., Khan, S. A., Septer, S., and Umar, S. (2016) Microbiome, Metabolome and Inflammatory Bowel Disease, *Microorganisms 4*.

25. Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., and Relman, D. A. (2005) Diversity of the human intestinal microbial flora, *Science 308*, 1635-1638.

26. Joossens, M., Huys, G., Cnockaert, M., De Preter, V., Verbeke, K., Rutgeerts, P., Vandamme, P., and Vermeire, S. (2011) Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives, *Gut 60*, 631-637.

27. Martinez, C., Antolin, M., Santos, J., Torrejon, A., Casellas, F., Borruel, N., Guarner, F., and Malagelada, J. R. (2008) Unstable composition of the fecal microbiota in ulcerative colitis during clinical remission, *The American journal of gastroenterology 103*, 643-648.

28. Frank, D. N., St Amand, A. L., Feldman, R. A., Boedeker, E. C., Harpaz, N., and Pace, N. R. (2007) Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases, *Proceedings of the National Academy of Sciences of the United States of America 104*, 13780-13785.

29. Meconi, S., Vercellone, A., Levillain, F., Payre, B., Al Saati, T., Capilla, F., Desreumaux, P., Darfeuille-Michaud, A., and Altare, F. (2007) Adherent-invasive Escherichia coli isolated from Crohn's disease patients induce granulomas in vitro, *Cellular microbiology 9*, 1252-1261.

30. Gaya, D. R., Russell, R. K., Nimmo, E. R., and Satsangi, J. (2006) New genes in inflammatory bowel disease: lessons for complex diseases?, *Lancet 367*, 1271-1284.

31. Liu, J. Z., van Sommeren, S., Huang, H., Ng, S. C., Alberts, R., Takahashi, A., Ripke, S., Lee, J. C., Jostins, L., Shah, T., Abedian, S., Cheon, J. H., Cho, J., Dayani, N. E., Franke, L., Fuyuno, Y., Hart, A., Juyal, R. C., Juyal, G., Kim, W. H., Morris, A. P., Poustchi, H., Newman, W. G., Midha, V., Orchard, T. R., Vahedi, H., Sood, A., Sung, J. Y., Malekzadeh, R., Westra, H. J., Yamazaki, K., Yang, S. K., International Multiple Sclerosis Genetics, C., International, I. B. D. G. C., Barrett, J. C., Alizadeh, B. Z., Parkes, M., Bk, T., Daly, M. J., Kubo, M., Anderson, C. A., and Weersma, R. K. (2015) Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations, *Nature genetics 47*, 979-986.

32. Manolio, T. A. (2010) Genomewide association studies and assessment of the risk of disease, *The New England journal of medicine 363*, 166-176.

33. Ogura, Y., Bonen, D. K., Inohara, N., Nicolae, D. L., Chen, F. F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R. H., Achkar, J. P., Brant, S. R., Bayless, T. M., Kirschner, B. S., Hanauer, S. B., Nunez, G., and Cho, J. H. (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease, *Nature 411*, 603-606.

34. Travassos, L. H., Carneiro, L. A., Ramjeet, M., Hussey, S., Kim, Y. G., Magalhaes, J. G., Yuan, L., Soares, F., Chea, E., Le Bourhis, L., Boneca, I. G., Allaoui, A., Jones, N. L., Nunez, G., Girardin, S. E., and Philpott, D. J. (2010) Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry, *Nature immunology 11*, 55-62.

35. Hampe, J., Franke, A., Rosenstiel, P., Till, A., Teuber, M., Huse, K., Albrecht, M., Mayr, G., De La Vega, F. M., Briggs, J., Gunther, S., Prescott, N. J., Onnie, C. M., Hasler, R., Sipos, B., Folsch, U. R., Lengauer, T., Platzer, M., Mathew, C. G., Krawczak, M., and Schreiber, S. (2007) A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1, *Nature genetics 39*, 207-211.

36. Homer, C. R., Richmond, A. L., Rebert, N. A., Achkar, J. P., and McDonald, C. (2010) ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis, *Gastroenterology 139*, 1630-1641, 1641 e1631-1632.
37. Brand, S. (2009) Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease, *Gut 58*, 1152-1167.
38. Amre, D. K., Mack, D. R., Morgan, K., Israel, D., Lambrette, P., Costea, I., Krupoves, A., Fegury, H., Dong, J., Grimard, G., Deslandres, C., Levy, E., and Seidman, E. G. (2009) Interleukin 10 (IL-10) gene variants and susceptibility for paediatric onset Crohn's disease, *Alimentary pharmacology & therapeutics 29*, 1025-1031.
39. Targan, S. R., and Karp, L. C. (2005) Defects in mucosal immunity leading to ulcerative colitis, *Immunological reviews 206*, 296-305.
40. Geremia, A., and Jewell, D. P. (2012) The IL-23/IL-17 pathway in inflammatory bowel disease, *Expert review of gastroenterology & hepatology 6*, 223-237.
41. Salim, S. Y., and Soderholm, J. D. (2011) Importance of disrupted intestinal barrier in inflammatory bowel diseases, *Inflammatory bowel diseases 17*, 362-381.
42. Wehkamp, J., Harder, J., Weichenthal, M., Mueller, O., Herrlinger, K. R., Fellermann, K., Schroeder, J. M., and Stange, E. F. (2003) Inducible and constitutive beta-defensins are differentially expressed in Crohn's disease and ulcerative colitis, *Inflammatory bowel diseases 9*, 215-223.
43. Medzhitov, R., and Janeway, C., Jr. (2000) Innate immunity, *The New England journal of medicine 343*, 338-344.
44. Abreu, M. T., Fukata, M., and Arditi, M. (2005) TLR signaling in the gut in health and disease, *Journal of immunology 174*, 4453-4460.
45. Wehkamp, J., Harder, J., Weichenthal, M., Schwab, M., Schaffeler, E., Schlee, M., Herrlinger, K. R., Stallmach, A., Noack, F., Fritz, P., Schroder, J. M., Bevins, C. L., Fellermann, K., and Stange, E. F. (2004) NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression, *Gut 53*, 1658-1664.
46. Abraham, C., and Cho, J. H. (2006) Functional consequences of NOD2 (CARD15) mutations, *Inflammatory bowel diseases 12*, 641-650.

47. Watanabe, T., Kitani, A., Murray, P. J., and Strober, W. (2004) NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses, *Nature immunology 5*, 800-808.

48. Noguchi, E., Homma, Y., Kang, X., Netea, M. G., and Ma, X. (2009) A Crohn's disease-associated NOD2 mutation suppresses transcription of human IL10 by inhibiting activity of the nuclear ribonucleoprotein hnRNP-A1, *Nature immunology 10*, 471-479.

49. Korn, T., Bettelli, E., Oukka, M., and Kuchroo, V. K. (2009) IL-17 and Th17 Cells, *Annual review of immunology 27*, 485-517.

50. Breese, E., Braegger, C. P., Corrigan, C. J., Walker-Smith, J. A., and MacDonald, T. T. (1993) Interleukin-2- and interferon-gamma-secreting T cells in normal and diseased human intestinal mucosa, *Immunology 78*, 127-131.

51. Heller, F., Florian, P., Bojarski, C., Richter, J., Christ, M., Hillenbrand, B., Mankertz, J., Gitter, A. H., Burgel, N., Fromm, M., Zeitz, M., Fuss, I., Strober, W., and Schulzke, J. D. (2005) Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution, *Gastroenterology 129*, 550-564.

52. Di Sabatino, A., Biancheri, P., Rovedatti, L., MacDonald, T. T., and Corazza, G. R. (2012) New pathogenic paradigms in inflammatory bowel disease, *Inflammatory bowel diseases 18*, 368-371.

53. Rovedatti, L., Kudo, T., Biancheri, P., Sarra, M., Knowles, C. H., Rampton, D. S., Corazza, G. R., Monteleone, G., Di Sabatino, A., and Macdonald, T. T. (2009) Differential regulation of interleukin 17 and interferon gamma production in inflammatory bowel disease, *Gut 58*, 1629-1636.

54. Kadivar, K., Ruchelli, E. D., Markowitz, J. E., Defelice, M. L., Strogatz, M. L., Kanzaria, M. M., Reddy, K. P., Baldassano, R. N., von Allmen, D., and Brown, K. A. (2004) Intestinal interleukin-13 in pediatric inflammatory bowel disease patients, *Inflammatory bowel diseases 10*, 593-598.

55. Wilson, M. S., Ramalingam, T. R., Rivollier, A., Shenderov, K., Mentink-Kane, M. M., Madala, S. K., Cheever, A. W., Artis, D., Kelsall, B. L., and Wynn, T. A. (2011) Colitis and intestinal inflammation in IL10-/- mice results from IL-13Ralpha2-mediated attenuation of IL-13 activity, *Gastroenterology 140*, 254-264.

56. Kobayashi, T., Okamoto, S., Hisamatsu, T., Kamada, N., Chinen, H., Saito, R., Kitazume, M. T., Nakazawa, A., Sugita, A., Koganei, K., Isobe, K., and Hibi, T. (2008) IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease, *Gut 57*, 1682-1689.

57. Sugihara, T., Kobori, A., Imaeda, H., Tsujikawa, T., Amagase, K., Takeuchi, K., Fujiyama, Y., and Andoh, A. (2010) The increased mucosal mRNA expressions of complement C3 and interleukin-17 in inflammatory bowel disease, *Clinical and experimental immunology 160*, 386-393.

58. Sieber, G., Herrmann, F., Zeitz, M., Teichmann, H., and Ruhl, H. (1984) Abnormalities of B-cell activation and immunoregulation in patients with Crohn's disease, *Gut 25*, 1255-1261.

59. Quinton, J. F., Sendid, B., Reumaux, D., Duthilleul, P., Cortot, A., Grandbastien, B., Charrier, G., Targan, S. R., Colombel, J. F., and Poulain, D. (1998) Anti-Saccharomyces cerevisiae mannan antibodies combined with antineutrophil cytoplasmic autoantibodies in inflammatory bowel disease: prevalence and diagnostic role, *Gut 42*, 788-791.

60. van Schaik, F. D., Oldenburg, B., Hart, A. R., Siersema, P. D., Lindgren, S., Grip, O., Teucher, B., Kaaks, R., Bergmann, M. M., Boeing, H., Carbonnel, F., Jantchou, P., Boutron-Ruault, M. C., Tjonneland, A., Olsen, A., Crowe, F. L., Peeters, P. H., van Oijen, M. G., and Bueno-de-Mesquita, H. B. (2013) Serological markers predict inflammatory bowel disease years before the diagnosis, *Gut 62*, 683-688.

61. Mizoguchi, E., Mizoguchi, A., Preffer, F. I., and Bhan, A. K. (2000) Regulatory role of mature B cells in a murine model of inflammatory bowel disease, *International immunology 12*, 597-605.

62. Olson, T. S., Bamias, G., Naganuma, M., Rivera-Nieves, J., Burcin, T. L., Ross, W., Morris, M. A., Pizarro, T. T., Ernst, P. B., Cominelli, F., and Ley, K. (2004) Expanded B cell population blocks regulatory T cells and exacerbates ileitis in a murine model of Crohn disease, *The Journal of clinical investigation 114*, 389-398.

63. LeBien, T. W., and Tedder, T. F. (2008) B lymphocytes: how they develop and function, *Blood 112*, 1570-1580.

64. Timmermans, W. M., van Laar, J. A., van der Houwen, T. B., Kamphuis, L. S., Bartol, S. J., Lam, K. H., Ouwendijk, R. J., Sparrow, M. P., Gibson, P. R., van Hagen, P. M., and van

Zelm, M. C. (2016) B-Cell Dysregulation in Crohn's Disease Is Partially Restored with Infliximab Therapy, *PloS one 11*, e0160103.

65. Williams, C., Panaccione, R., Ghosh, S., and Rioux, K. (2011) Optimizing clinical use of mesalazine (5-aminosalicylic acid) in inflammatory bowel disease, *Therapeutic advances in gastroenterology 4*, 237-248.
66. Yamamoto-Furusho, J. K., Penaloza-Coronel, A., Sanchez-Munoz, F., Barreto-Zuniga, R., and Dominguez-Lopez, A. (2011) Peroxisome proliferator-activated receptor-gamma (PPAR-gamma) expression is downregulated in patients with active ulcerative colitis, *Inflammatory bowel diseases 17*, 680-681.
67. Kuenzig, M. E., Rezaie, A., Seow, C. H., Otley, A. R., Steinhart, A. H., Griffiths, A. M., Kaplan, G. G., and Benchimol, E. I. (2014) Budesonide for maintenance of remission in Crohn's disease, *The Cochrane database of systematic reviews*, CD002913.
68. Markowitz, J., Grancher, K., Kohn, N., and Daum, F. (2002) Immunomodulatory therapy for pediatric inflammatory bowel disease: changing patterns of use, 1990-2000, *The American journal of gastroenterology 97*, 928-932.
69. Wessels, J. A., Huizinga, T. W., and Guchelaar, H. J. (2008) Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis, *Rheumatology 47*, 249-255.
70. Brody, M., Bohm, I., and Bauer, R. (1993) Mechanism of action of methotrexate: experimental evidence that methotrexate blocks the binding of interleukin 1 beta to the interleukin 1 receptor on target cells, *European journal of clinical chemistry and clinical biochemistry : journal of the Forum of European Clinical Chemistry Societies 31*, 667-674.
71. van Dullemen, H. M., van Deventer, S. J., Hommes, D. W., Bijl, H. A., Jansen, J., Tytgat, G. N., and Woody, J. (1995) Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2), *Gastroenterology 109*, 129-135.
72. Poggioli, G., Laureti, S., Campieri, M., Pierangeli, F., Gionchetti, P., Ugolini, F., Gentilini, L., Bazzi, P., Rizzello, F., and Coscia, M. (2007) Infliximab in the treatment of Crohn's disease, *Therapeutics and clinical risk management 3*, 301-308.
73. Levin, A., and Shibolet, O. (2008) Infliximab in ulcerative colitis, *Biologics : targets & therapy 2*, 379-388.

74. Laroui, H., Ingersoll, S. A., Liu, H. C., Baker, M. T., Ayyadurai, S., Charania, M. A., Laroui, F., Yan, Y., Sitaraman, S. V., and Merlin, D. (2012) Dextran sodium sulfate (DSS) induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon, *PloS one 7*, e32084.
75. Perse, M., and Cerar, A. (2012) Dextran sodium sulphate colitis mouse model: traps and tricks, *Journal of biomedicine & biotechnology 2012*, 718617.
76. Yan, Y., Kolachala, V., Dalmasso, G., Nguyen, H., Laroui, H., Sitaraman, S. V., and Merlin, D. (2009) Temporal and spatial analysis of clinical and molecular parameters in dextran sodium sulfate induced colitis, *PloS one 4*, e6073.
77. Dou, W., Zhang, J., Sun, A., Zhang, E., Ding, L., Mukherjee, S., Wei, X., Chou, G., Wang, Z. T., and Mani, S. (2013) Protective effect of naringenin against experimental colitis via suppression of Toll-like receptor 4/NF-kappaB signalling, *The British journal of nutrition 110*, 599-608.
78. Low, D., Nguyen, D. D., and Mizoguchi, E. (2013) Animal models of ulcerative colitis and their application in drug research, *Drug design, development and therapy 7*, 1341-1357.
79. Araki, Y., Mukaisyo, K., Sugihara, H., Fujiyama, Y., and Hattori, T. (2010) Increased apoptosis and decreased proliferation of colonic epithelium in dextran sulfate sodium-induced colitis in mice, *Oncology reports 24*, 869-874.
80. Gordon, M., El-Kalla, M., Zhao, Y., Fiteih, Y., Law, J., Volodko, N., Anwar-Mohamed, A., El-Kadi, A. O., Liu, L., Odenbach, J., Thiesen, A., Onyskiw, C., Ghazaleh, H. A., Park, J., Lee, S. B., Yu, V. C., Fernandez-Patron, C., Alexander, R. T., Wine, E., and Baksh, S. (2013) The tumor suppressor gene, RASSF1A, is essential for protection against inflammation -induced injury, *PloS one 8*, e75483.
81. Unutmaz, D., and Pulendran, B. (2009) The gut feeling of Treg cells: IL-10 is the silver lining during colitis, *Nature immunology 10*, 1141-1143.
82. Kiesler, P., Fuss, I. J., and Strober, W. (2015) Experimental Models of Inflammatory Bowel Diseases, *Cellular and molecular gastroenterology and hepatology 1*, 154-170.
83. Marlow, G. J., van Gent, D., and Ferguson, L. R. (2013) Why interleukin-10 supplementation does not work in Crohn's disease patients, *World journal of gastroenterology 19*, 3931-3941.

84. Eckmann, L., and Karin, M. (2005) NOD2 and Crohn's disease: loss or gain of function?, *Immunity 22*, 661-667.
85. Kobayashi, K. S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nunez, G., and Flavell, R. A. (2005) Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract, *Science 307*, 731-734.
86. Hugot, J. P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J. P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C. A., Gassull, M., Binder, V., Finkel, Y., Cortot, A., Modigliani, R., Laurent-Puig, P., Gower-Rousseau, C., Macry, J., Colombel, J. F., Sahbatou, M., and Thomas, G. (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease, *Nature 411*, 599-603.
87. Maeda, S., Hsu, L. C., Liu, H., Bankston, L. A., Iimura, M., Kagnoff, M. F., Eckmann, L., and Karin, M. (2005) Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing, *Science 307*, 734-738.
88. Zhang, M., Sun, K., Wu, Y., Yang, Y., Tso, P., and Wu, Z. (2017) Interactions between Intestinal Microbiota and Host Immune Response in Inflammatory Bowel Disease, *Frontiers in immunology 8*, 942.
89. MMP-9 in Inflammatory Bowel Disease (IBD) and in Metastatic Cancers
90. Pasparakis, M. (2008) IKK/NF-kappaB signaling in intestinal epithelial cells controls immune homeostasis in the gut, *Mucosal immunology 1 Suppl 1*, S54-57.
91. Richter, A. M., Pfeifer, G. P., and Dammann, R. H. (2009) The RASSF proteins in cancer; from epigenetic silencing to functional characterization, *Biochimica et biophysica acta 1796*, 114-128.
92. Gordon, M., and Baksh, S. (2011) RASSF1A: Not a prototypical Ras effector, *Small GTPases 2*, 148-157.
93. Reeves, M. E., Baldwin, S. W., Baldwin, M. L., Chen, S. T., Moretz, J. M., Aragon, R. J., Li, X., Strong, D. D., Mohan, S., and Amaar, Y. G. (2010) Ras-association domain family 1C protein promotes breast cancer cell migration and attenuates apoptosis, *BMC cancer 10*, 562.
94. Avruch, J., Xavier, R., Bardeesy, N., Zhang, X. F., Praskova, M., Zhou, D., and Xia, F. (2009) Rassf family of tumor suppressor polypeptides, *The Journal of biological chemistry 284*, 11001-11005.

95. Hamilton, G., Yee, K. S., Scrace, S., and O'Neill, E. (2009) ATM regulates a RASSF1A-dependent DNA damage response, *Current biology : CB 19*, 2020-2025.
96. Foley, C. J., Freedman, H., Choo, S. L., Onyskiw, C., Fu, N. Y., Yu, V. C., Tuszynski, J., Pratt, J. C., and Baksh, S. (2008) Dynamics of RASSF1A/MOAP-1 association with death receptors, *Molecular and cellular biology 28*, 4520-4535.
97. El-Kalla, M., Onyskiw, C., and Baksh, S. (2010) Functional importance of RASSF1A microtubule localization and polymorphisms, *Oncogene 29*, 5729-5740.
98. Dallol, A., Agathanggelou, A., Fenton, S. L., Ahmed-Choudhury, J., Hesson, L., Vos, M. D., Clark, G. J., Downward, J., Maher, E. R., and Latif, F. (2004) RASSF1A interacts with microtubule-associated proteins and modulates microtubule dynamics, *Cancer research 64*, 4112-4116.
99. Vos, M. D., Martinez, A., Elam, C., Dallol, A., Taylor, B. J., Latif, F., and Clark, G. J. (2004) A role for the RASSF1A tumor suppressor in the regulation of tubulin polymerization and genomic stability, *Cancer research 64*, 4244-4250.
100. Dallol, A., Agathanggelou, A., Tommasi, S., Pfeifer, G. P., Maher, E. R., and Latif, F. (2005) Involvement of the RASSF1A tumor suppressor gene in controlling cell migration, *Cancer research 65*, 7653-7659.
101. Xie, R., Nguyen, S., McKeehan, K., Wang, F., McKeehan, W. L., and Liu, L. (2011) Microtubule-associated protein 1S (MAP1S) bridges autophagic components with microtubules and mitochondria to affect autophagosomal biogenesis and degradation, *The Journal of biological chemistry 286*, 10367-10377.
102. Baldin, V., Lukas, J., Marcote, M. J., Pagano, M., and Draetta, G. (1993) Cyclin D1 is a nuclear protein required for cell cycle progression in G1, *Genes & development 7*, 812-821.
103. Song, M. S., Chang, J. S., Song, S. J., Yang, T. H., Lee, H., and Lim, D. S. (2005) The centrosomal protein RAS association domain family protein 1A (RASSF1A)-binding protein 1 regulates mitotic progression by recruiting RASSF1A to spindle poles, *The Journal of biological chemistry 280*, 3920-3927.
104. Song, M. S., Song, S. J., Ayad, N. G., Chang, J. S., Lee, J. H., Hong, H. K., Lee, H., Choi, N., Kim, J., Kim, H., Kim, J. W., Choi, E. J., Kirschner, M. W., and Lim, D. S. (2004) The

tumour suppressor RASSF1A regulates mitosis by inhibiting the APC-Cdc20 complex, *Nature cell biology 6*, 129-137.

105. Song, S. J., Song, M. S., Kim, S. J., Kim, S. Y., Kwon, S. H., Kim, J. G., Calvisi, D. F., Kang, D., and Lim, D. S. (2009) Aurora A regulates prometaphase progression by inhibiting the ability of RASSF1A to suppress APC-Cdc20 activity, *Cancer research 69*, 2314-2323.
106. Shivakumar, L., Minna, J., Sakamaki, T., Pestell, R., and White, M. A. (2002) The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation, *Molecular and cellular biology 22*, 4309-4318.
107. Whang, Y. M., Kim, Y. H., Kim, J. S., and Yoo, Y. D. (2005) RASSF1A suppresses the c-Jun-NH2-kinase pathway and inhibits cell cycle progression, *Cancer research 65*, 3682-3690.
108. Danial, N. N., and Korsmeyer, S. J. (2004) Cell death: critical control points, *Cell 116*, 205-219.
109. Thorburn, A. (2004) Death receptor-induced cell killing, *Cellular signalling 16*, 139-144.
110. Kroemer, G. (2002) Introduction: mitochondrial control of apoptosis, *Biochimie 84*, 103-104.
111. Amin, K. S., and Banerjee, P. P. (2012) The cellular functions of RASSF1A and its inactivation in prostate cancer, *Journal of carcinogenesis 11*, 3.
112. Rawat, S. J., Creasy, C. L., Peterson, J. R., and Chernoff, J. (2013) The tumor suppressor Mst1 promotes changes in the cellular redox state by phosphorylation and inactivation of peroxiredoxin-1 protein, *The Journal of biological chemistry 288*, 8762-8771.
113. Dittfeld, C., Richter, A. M., Steinmann, K., Klagge-Ulonska, A., and Dammann, R. H. (2012) The SARAH Domain of RASSF1A and Its Tumor Suppressor Function, *Molecular biology international 2012*, 196715.
114. Guo, C., Tommasi, S., Liu, L., Yee, J. K., Dammann, R., and Pfeifer, G. P. (2007) RASSF1A is part of a complex similar to the Drosophila Hippo/Salvador/Lats tumor-suppressor network, *Current biology : CB 17*, 700-705.
115. Matallanas, D., Romano, D., Yee, K., Meissl, K., Kucerova, L., Piazzolla, D., Baccarini, M., Vass, J. K., Kolch, W., and O'Neill, E. (2007) RASSF1A elicits apoptosis through an

MST2 pathway directing proapoptotic transcription by the p73 tumor suppressor protein, *Molecular cell 27*, 962-975.

116. Oh, H. J., Lee, K. K., Song, S. J., Jin, M. S., Song, M. S., Lee, J. H., Im, C. R., Lee, J. O., Yonehara, S., and Lim, D. S. (2006) Role of the tumor suppressor RASSF1A in Mst1-mediated apoptosis, *Cancer research 66*, 2562-2569.
117. Baksh, S., Tommasi, S., Fenton, S., Yu, V. C., Martins, L. M., Pfeifer, G. P., Latif, F., Downward, J., and Neel, B. G. (2005) The tumor suppressor RASSF1A and MAP-1 link death receptor signaling to Bax conformational change and cell death, *Molecular cell 18*, 637-650.
118. Dammann, R., Schagdarsurengin, U., Seidel, C., Strunnikova, M., Rastetter, M., Baier, K., and Pfeifer, G. P. (2005) The tumor suppressor RASSF1A in human carcinogenesis: an update, *Histology and histopathology 20*, 645-663.
119. Pfeifer, G. P., Yoon, J. H., Liu, L., Tommasi, S., Wilczynski, S. P., and Dammann, R. (2002) Methylation of the RASSF1A gene in human cancers, *Biological chemistry 383*, 907-914.
120. Miranda, E., Destro, A., Malesci, A., Balladore, E., Bianchi, P., Baryshnikova, E., Franchi, G., Morenghi, E., Laghi, L., Gennari, L., and Roncalli, M. (2006) Genetic and epigenetic changes in primary metastatic and nonmetastatic colorectal cancer, *British journal of cancer 95*, 1101-1107.
121. Abouzeid, H. E., Kassem, A. M., Abdel Wahab, A. H., El-mezayen, H. A., Sharad, H., and Abdel Rahman, S. (2011) Promoter hypermethylation of RASSF1A, MGMT, and HIC-1 genes in benign and malignant colorectal tumors, *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine 32*, 845-852.
122. Malpeli, G., Amato, E., Dandrea, M., Fumagalli, C., Debattisti, V., Boninsegna, L., Pelosi, G., Falconi, M., and Scarpa, A. (2011) Methylation-associated down-regulation of RASSF1A and up-regulation of RASSF1C in pancreatic endocrine tumors, *BMC cancer 11*, 351.
123. Tommasi, S., Dammann, R., Zhang, Z., Wang, Y., Liu, L., Tsark, W. M., Wilczynski, S. P., Li, J., You, M., and Pfeifer, G. P. (2005) Tumor susceptibility of Rassf1a knockout mice, *Cancer research 65*, 92-98.

124. Tamm, C., Bower, N., and Anneren, C. (2011) Regulation of mouse embryonic stem cell self-renewal by a Yes-YAP-TEAD2 signaling pathway downstream of LIF, *Journal of cell science 124*, 1136-1144.

125. Levy, D., Adamovich, Y., Reuven, N., and Shaul, Y. (2008) Yap1 phosphorylation by c-Abl is a critical step in selective activation of proapoptotic genes in response to DNA damage, *Molecular cell 29*, 350-361.

126. Goldberg, Z., Vogt Sionov, R., Berger, M., Zwang, Y., Perets, R., Van Etten, R. A., Oren, M., Taya, Y., and Haupt, Y. (2002) Tyrosine phosphorylation of Mdm2 by c-Abl: implications for p53 regulation, *The EMBO journal 21*, 3715-3727.

127. Zhang, H., Wu, S., and Xing, D. (2011) YAP accelerates Abeta(25-35)-induced apoptosis through upregulation of Bax expression by interaction with p73, *Apoptosis : an international journal on programmed cell death 16*, 808-821.

128. Tigno-Aranjuez, J. T., Bai, X., and Abbott, D. W. (2013) A discrete ubiquitin-mediated network regulates the strength of NOD2 signaling, *Molecular and cellular biology 33*, 146-158.

129. Pefani, D. E., Pankova, D., Abraham, A. G., Grawenda, A. M., Vlahov, N., Scrace, S., and E, O. N. (2016) TGF-beta Targets the Hippo Pathway Scaffold RASSF1A to Facilitate YAP/SMAD2 Nuclear Translocation, *Molecular cell 63*, 156-166.

130. Kawai, T., and Akira, S. (2009) The roles of TLRs, RLRs and NLRs in pathogen recognition, *International immunology 21*, 317-337.

131. Franchi, L., Warner, N., Viani, K., and Nunez, G. (2009) Function of Nod-like receptors in microbial recognition and host defense, *Immunological reviews 227*, 106-128.

132. Proell, M., Riedl, S. J., Fritz, J. H., Rojas, A. M., and Schwarzenbacher, R. (2008) The Nod-like receptor (NLR) family: a tale of similarities and differences, *PloS one 3*, e2119.

133. Ting, J. P., Lovering, R. C., Alnemri, E. S., Bertin, J., Boss, J. M., Davis, B. K., Flavell, R. A., Girardin, S. E., Godzik, A., Harton, J. A., Hoffman, H. M., Hugot, J. P., Inohara, N., Mackenzie, A., Maltais, L. J., Nunez, G., Ogura, Y., Otten, L. A., Philpott, D., Reed, J. C., Reith, W., Schreiber, S., Steimle, V., and Ward, P. A. (2008) The NLR gene family: a standard nomenclature, *Immunity 28*, 285-287.

134. Inohara, N., Ogura, Y., Chen, F. F., Muto, A., and Nunez, G. (2001) Human Nod1 confers responsiveness to bacterial lipopolysaccharides, *The Journal of biological chemistry 276*, 2551-2554.

135. Ogura, Y., Inohara, N., Benito, A., Chen, F. F., Yamaoka, S., and Nunez, G. (2001) Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB, *The Journal of biological chemistry 276*, 4812-4818.

136. Inohara, Chamaillard, McDonald, C., and Nunez, G. (2005) NOD-LRR proteins: role in host-microbial interactions and inflammatory disease, *Annual review of biochemistry 74*, 355-383.

137. Chen, G., Shaw, M. H., Kim, Y. G., and Nunez, G. (2009) NOD-like receptors: role in innate immunity and inflammatory disease, *Annual review of pathology 4*, 365-398.

138. Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., Valvano, M. A., Foster, S. J., Mak, T. W., Nunez, G., and Inohara, N. (2003) An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid, *Nature immunology 4*, 702-707.

139. Girardin, S. E., Boneca, I. G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D. J., and Sansonetti, P. J. (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection, *The Journal of biological chemistry 278*, 8869-8872.

140. Mohanan, V., and Grimes, C. L. (2014) The molecular chaperone HSP70 binds to and stabilizes NOD2, an important protein involved in Crohn disease, *The Journal of biological chemistry 289*, 18987-18998.

141. Hahn, J. S. (2005) Regulation of Nod1 by Hsp90 chaperone complex, *FEBS letters 579*, 4513-4519.

142. Lee, K. H., Biswas, A., Liu, Y. J., and Kobayashi, K. S. (2012) Proteasomal degradation of Nod2 protein mediates tolerance to bacterial cell wall components, *The Journal of biological chemistry 287*, 39800-39811.

143. Park, J. H., Kim, Y. G., McDonald, C., Kanneganti, T. D., Hasegawa, M., Body-Malapel, M., Inohara, N., and Nunez, G. (2007) RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs, *Journal of immunology 178*, 2380-2386.

144. Hasegawa, M., Fujimoto, Y., Lucas, P. C., Nakano, H., Fukase, K., Nunez, G., and Inohara, N. (2008) A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation, *The EMBO journal 27*, 373-383.

145. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB, *Nature 388*, 548-554.

146. Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998) Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation, *Cell 93*, 1231-1240.

147. Shaw, M. H., Reimer, T., Kim, Y. G., and Nunez, G. (2008) NOD-like receptors (NLRs): bona fide intracellular microbial sensors, *Current opinion in immunology 20*, 377-382.

148. Vandenabeele, P., and Bertrand, M. J. (2012) The role of the IAP E3 ubiquitin ligases in regulating pattern-recognition receptor signalling, *Nature reviews. Immunology 12*, 833-844.

149. Verma, R., Verma, N., and Paul, J. (2013) Expression of inflammatory genes in the colon of ulcerative colitis patients varies with activity both at the mRNA and protein level, *European cytokine network 24*, 130-138.

150. Ferreira, C. M., Vieira, A. T., Vinolo, M. A., Oliveira, F. A., Curi, R., and Martins Fdos, S. (2014) The central role of the gut microbiota in chronic inflammatory diseases, *Journal of immunology research 2014*, 689492.

151. Rehman, A., Sina, C., Gavrilova, O., Hasler, R., Ott, S., Baines, J. F., Schreiber, S., and Rosenstiel, P. (2011) Nod2 is essential for temporal development of intestinal microbial communities, *Gut 60*, 1354-1362.

152. Biswas, A., Petnicki-Ocwieja, T., and Kobayashi, K. S. (2012) Nod2: a key regulator linking microbiota to intestinal mucosal immunity, *Journal of molecular medicine 90*, 15-24.

153. Ramanan, D., Tang, M. S., Bowcutt, R., Loke, P., and Cadwell, K. (2014) Bacterial sensor Nod2 prevents inflammation of the small intestine by restricting the expansion of the commensal Bacteroides vulgatus, *Immunity 41*, 311-324.

154. Petnicki-Ocwieja, T., Hrncir, T., Liu, Y. J., Biswas, A., Hudcovic, T., Tlaskalova-Hogenova, H., and Kobayashi, K. S. (2009) Nod2 is required for the regulation of

commensal microbiota in the intestine, *Proceedings of the National Academy of Sciences of the United States of America 106*, 15813-15818.

155. Frank, D. N., Robertson, C. E., Hamm, C. M., Kpadeh, Z., Zhang, T., Chen, H., Zhu, W., Sartor, R. B., Boedeker, E. C., Harpaz, N., Pace, N. R., and Li, E. (2011) Disease phenotype and genotype are associated with shifts in intestinal-associated microbiota in inflammatory bowel diseases, *Inflammatory bowel diseases 17*, 179-184.

156. Juyal, G., Amre, D., Midha, V., Sood, A., Seidman, E., and Thelma, B. K. (2007) Evidence of allelic heterogeneity for associations between the NOD2/CARD15 gene and ulcerative colitis among North Indians, *Alimentary pharmacology & therapeutics 26*, 1325-1332.

157. Walker, D. G., Williams, H. R., Bancil, A. S., Rai, P., Pantelidis, P., Chambers, J., Kooner, J. S., Sato, H., and Orchard, T. R. (2013) Ethnicity differences in genetic susceptibility to ulcerative colitis: a comparison of Indian asians and white northern Europeans, *Inflammatory bowel diseases 19*, 2888-2894.

158. Hold, G. L., Smith, M., Grange, C., Watt, E. R., El-Omar, E. M., and Mukhopadhya, I. (2014) Role of the gut microbiota in inflammatory bowel disease pathogenesis: what have we learnt in the past 10 years?, *World journal of gastroenterology 20*, 1192-1210.

159. Russell, R. K., Nimmo, E. R., and Satsangi, J. (2004) Molecular genetics of Crohn's disease, *Current opinion in genetics & development 14*, 264-270.

160. Hugot, J. P., Zaccaria, I., Cavanaugh, J., Yang, H., Vermeire, S., Lappalainen, M., Schreiber, S., Annese, V., Jewell, D. P., Fowler, E. V., Brant, S. R., Silverberg, M. S., Cho, J., Rioux, J. D., Satsangi, J., Parkes, M., and Consortium, I. B. D. I. G. (2007) Prevalence of CARD15/NOD2 mutations in Caucasian healthy people, *The American journal of gastroenterology 102*, 1259-1267.

161. Kim, Y. G., Shaw, M. H., Warner, N., Park, J. H., Chen, F., Ogura, Y., and Nunez, G. (2011) Cutting edge: Crohn's disease-associated Nod2 mutation limits production of proinflammatory cytokines to protect the host from Enterococcus faecalis-induced lethality, *Journal of immunology 187*, 2849-2852.

162. Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., Fukase, K., Inamura, S., Kusumoto, S., Hashimoto, M., Foster, S. J., Moran, A. P., Fernandez-Luna, J. L., and Nunez, G. (2003) Host recognition of bacterial muramyl dipeptide mediated

through NOD2. Implications for Crohn's disease, *The Journal of biological chemistry 278*, 5509-5512.

163. Wehkamp, J., Salzman, N. H., Porter, E., Nuding, S., Weichenthal, M., Petras, R. E., Shen, B., Schaeffeler, E., Schwab, M., Linzmeier, R., Feathers, R. W., Chu, H., Lima, H., Jr., Fellermann, K., Ganz, T., Stange, E. F., and Bevins, C. L. (2005) Reduced Paneth cell alpha-defensins in ileal Crohn's disease, *Proceedings of the National Academy of Sciences of the United States of America 102*, 18129-18134.
164. Simms, L. A., Doecke, J. D., Walsh, M. D., Huang, N., Fowler, E. V., and Radford-Smith, G. L. (2008) Reduced alpha-defensin expression is associated with inflammation and not NOD2 mutation status in ileal Crohn's disease, *Gut 57*, 903-910.
165. Sorbara, M. T., Ellison, L. K., Ramjeet, M., Travassos, L. H., Jones, N. L., Girardin, S. E., and Philpott, D. J. (2013) The protein ATG16L1 suppresses inflammatory cytokines induced by the intracellular sensors Nod1 and Nod2 in an autophagy-independent manner, *Immunity 39*, 858-873.
166. Wolfkamp, S. C., Verseyden, C., Vogels, E. W., Meisner, S., Boonstra, K., Peters, C. P., Stokkers, P. C., and te Velde, A. A. (2014) ATG16L1 and NOD2 polymorphisms enhance phagocytosis in monocytes of Crohn's disease patients, *World journal of gastroenterology 20*, 2664-2672.
167. https://www.invivogen.com/review-nlr. Nod-Like Receptors.
168. He, C., and Klionsky, D. J. (2009) Regulation mechanisms and signaling pathways of autophagy, *Annual review of genetics 43*, 67-93.
169. Arstila, A. U., and Trump, B. F. (1968) Studies on cellular autophagocytosis. The formation of autophagic vacuoles in the liver after glucagon administration, *The American journal of pathology 53*, 687-733.
170. Li, W. W., Li, J., and Bao, J. K. (2012) Microautophagy: lesser-known self-eating, *Cellular and molecular life sciences : CMLS 69*, 1125-1136.
171. Mizushima, N., and Komatsu, M. (2011) Autophagy: renovation of cells and tissues, *Cell 147*, 728-741.
172. Kaushik, S., and Cuervo, A. M. (2012) Chaperone-mediated autophagy: a unique way to enter the lysosome world, *Trends in cell biology 22*, 407-417.

173. Kroemer, G., Marino, G., and Levine, B. (2010) Autophagy and the integrated stress response, *Molecular cell 40*, 280-293.
174. Weidberg, H., Shvets, E., and Elazar, Z. (2011) Biogenesis and cargo selectivity of autophagosomes, *Annual review of biochemistry 80*, 125-156.
175. Sumpter, R., Jr., and Levine, B. (2010) Autophagy and innate immunity: triggering, targeting and tuning, *Seminars in cell & developmental biology 21*, 699-711.
176. Matsuda, C., Ito, T., Song, J., Mizushima, T., Tamagawa, H., Kai, Y., Hamanaka, Y., Inoue, M., Nishida, T., Matsuda, H., and Sawa, Y. (2007) Therapeutic effect of a new immunosuppressive agent, everolimus, on interleukin-10 gene-deficient mice with colitis, *Clinical and experimental immunology 148*, 348-359.
177. Singletary, K., and Milner, J. (2008) Diet, autophagy, and cancer: a review, *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 17*, 1596-1610.
178. Dumortier, J., Lapalus, M. G., Guillaud, O., Poncet, G., Gagnieu, M. C., Partensky, C., and Scoazec, J. Y. (2008) Everolimus for refractory Crohn's disease: a case report, *Inflammatory bowel diseases 14*, 874-877.
179. Reinisch, W., Panes, J., Lemann, M., Schreiber, S., Feagan, B., Schmidt, S., Sturniolo, G. C., Mikhailova, T., Alexeeva, O., Sanna, L., Haas, T., Korom, S., and Mayer, H. (2008) A multicenter, randomized, double-blind trial of everolimus versus azathioprine and placebo to maintain steroid-induced remission in patients with moderate-to-severe active Crohn's disease, *The American journal of gastroenterology 103*, 2284-2292.
180. Wang, T. T., Dabbas, B., Laperriere, D., Bitton, A. J., Soualhine, H., Tavera-Mendoza, L. E., Dionne, S., Servant, M. J., Bitton, A., Seidman, E. G., Mader, S., Behr, M. A., and White, J. H. (2010) Direct and indirect induction by 1,25-dihydroxyvitamin D3 of the NOD2/CARD15-defensin beta2 innate immune pathway defective in Crohn disease, *The Journal of biological chemistry 285*, 2227-2231.
181. Dresner-Pollak, R., Ackerman, Z., Eliakim, R., Karban, A., Chowers, Y., and Fidder, H. H. (2004) The BsmI vitamin D receptor gene polymorphism is associated with ulcerative colitis in Jewish Ashkenazi patients, *Genetic testing 8*, 417-420.

182. Naderi, N., Farnood, A., Habibi, M., Derakhshan, F., Balaii, H., Motahari, Z., Agah, M. R., Firouzi, F., Rad, M. G., Aghazadeh, R., Zojaji, H., and Zali, M. R. (2008) Association of vitamin D receptor gene polymorphisms in Iranian patients with inflammatory bowel disease, *Journal of gastroenterology and hepatology 23*, 1816-1822.

183. Caro, L. H., Plomp, P. J., Wolvetang, E. J., Kerkhof, C., and Meijer, A. J. (1988) 3-Methyladenine, an inhibitor of autophagy, has multiple effects on metabolism, *European journal of biochemistry 175*, 325-329.

184. Massey, D. C., and Parkes, M. (2007) Genome-wide association scanning highlights two autophagy genes, ATG16L1 and IRGM, as being significantly associated with Crohn's disease, *Autophagy 3*, 649-651.

185. Simmons, J. D., Mullighan, C., Welsh, K. I., and Jewell, D. P. (2000) Vitamin D receptor gene polymorphism: association with Crohn's disease susceptibility, *Gut 47*, 211-214.

186. Kuester, D., Guenther, T., Biesold, S., Hartmann, A., Bataille, F., Ruemmele, P., Peters, B., Meyer, F., Schubert, D., Bohr, U. R., Malfertheiner, P., Lippert, H., Silver, A. R., Roessner, A., and Schneider-Stock, R. (2010) Aberrant methylation of DAPK in long-standing ulcerative colitis and ulcerative colitis-associated carcinoma, *Pathology, research and practice 206*, 616-624.

187. Henckaerts, L., Cleynen, I., Brinar, M., John, J. M., Van Steen, K., Rutgeerts, P., and Vermeire, S. (2011) Genetic variation in the autophagy gene ULK1 and risk of Crohn's disease, *Inflammatory bowel diseases 17*, 1392-1397.

188. Cummings, J. R., Cooney, R., Pathan, S., Anderson, C. A., Barrett, J. C., Beckly, J., Geremia, A., Hancock, L., Guo, C., Ahmad, T., Cardon, L. R., and Jewell, D. P. (2007) Confirmation of the role of ATG16L1 as a Crohn's disease susceptibility gene, *Inflammatory bowel diseases 13*, 941-946.

189. Barrett, J. C., Hansoul, S., Nicolae, D. L., Cho, J. H., Duerr, R. H., Rioux, J. D., Brant, S. R., Silverberg, M. S., Taylor, K. D., Barmada, M. M., Bitton, A., Dassopoulos, T., Datta, L. W., Green, T., Griffiths, A. M., Kistner, E. O., Murtha, M. T., Regueiro, M. D., Rotter, J. I., Schumm, L. P., Steinhart, A. H., Targan, S. R., Xavier, R. J., Consortium, N. I. G., Libioulle, C., Sandor, C., Lathrop, M., Belaiche, J., Dewit, O., Gut, I., Heath, S., Laukens, D., Mni, M., Rutgeerts, P., Van Gossum, A., Zelenika, D., Franchimont, D., Hugot, J. P., de Vos, M., Vermeire, S., Louis, E., Belgian-French, I. B. D. C., Wellcome Trust Case

Control, C., Cardon, L. R., Anderson, C. A., Drummond, H., Nimmo, E., Ahmad, T., Prescott, N. J., Onnie, C. M., Fisher, S. A., Marchini, J., Ghori, J., Bumpstead, S., Gwilliam, R., Tremelling, M., Deloukas, P., Mansfield, J., Jewell, D., Satsangi, J., Mathew, C. G., Parkes, M., Georges, M., and Daly, M. J. (2008) Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease, *Nature genetics 40*, 955-962.

190. Fritz, T., Niederreiter, L., Adolph, T., Blumberg, R. S., and Kaser, A. (2011) Crohn's disease: NOD2, autophagy and ER stress converge, *Gut 60*, 1580-1588.

191. Fujita, N., Itoh, T., Omori, H., Fukuda, M., Noda, T., and Yoshimori, T. (2008) The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy, *Molecular biology of the cell 19*, 2092-2100.

192. Rioux, J. D., Xavier, R. J., Taylor, K. D., Silverberg, M. S., Goyette, P., Huett, A., Green, T., Kuballa, P., Barmada, M. M., Datta, L. W., Shugart, Y. Y., Griffiths, A. M., Targan, S. R., Ippoliti, A. F., Bernard, E. J., Mei, L., Nicolae, D. L., Regueiro, M., Schumm, L. P., Steinhart, A. H., Rotter, J. I., Duerr, R. H., Cho, J. H., Daly, M. J., and Brant, S. R. (2007) Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis, *Nature genetics 39*, 596-604.

193. Hampe, J., Schreiber, S., Shaw, S. H., Lau, K. F., Bridger, S., Macpherson, A. J., Cardon, L. R., Sakul, H., Harris, T. J., Buckler, A., Hall, J., Stokkers, P., van Deventer, S. J., Nurnberg, P., Mirza, M. M., Lee, J. C., Lennard-Jones, J. E., Mathew, C. G., and Curran, M. E. (1999) A genomewide analysis provides evidence for novel linkages in inflammatory bowel disease in a large European cohort, *American journal of human genetics 64*, 808-816.

194. Fujita, N., Saitoh, T., Kageyama, S., Akira, S., Noda, T., and Yoshimori, T. (2009) Differential involvement of Atg16L1 in Crohn disease and canonical autophagy: analysis of the organization of the Atg16L1 complex in fibroblasts, *The Journal of biological chemistry 284*, 32602-32609.

195. Kuballa, P., Huett, A., Rioux, J. D., Daly, M. J., and Xavier, R. J. (2008) Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant, *PloS one 3*, e3391.

196. Saitoh, T., Fujita, N., Jang, M. H., Uematsu, S., Yang, B. G., Satoh, T., Omori, H., Noda, T., Yamamoto, N., Komatsu, M., Tanaka, K., Kawai, T., Tsujimura, T., Takeuchi, O.,

Yoshimori, T., and Akira, S. (2008) Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production, *Nature 456*, 264-268.

197. Plantinga, T. S., Crisan, T. O., Oosting, M., van de Veerdonk, F. L., de Jong, D. J., Philpott, D. J., van der Meer, J. W., Girardin, S. E., Joosten, L. A., and Netea, M. G. (2011) Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2, *Gut 60*, 1229-1235.
198. Cadwell, K., Liu, J. Y., Brown, S. L., Miyoshi, H., Loh, J., Lennerz, J. K., Kishi, C., Kc, W., Carrero, J. A., Hunt, S., Stone, C. D., Brunt, E. M., Xavier, R. J., Sleckman, B. P., Li, E., Mizushima, N., Stappenbeck, T. S., and Virgin, H. W. t. (2008) A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells, *Nature 456*, 259-263.
199. Schaffler, A., and Herfarth, H. (2005) Creeping fat in Crohn's disease: travelling in a creeper lane of research?, *Gut 54*, 742-744.
200. Lecine, P., Esmiol, S., Metais, J. Y., Nicoletti, C., Nourry, C., McDonald, C., Nunez, G., Hugot, J. P., Borg, J. P., and Ollendorff, V. (2007) The NOD2-RICK complex signals from the plasma membrane, *The Journal of biological chemistry 282*, 15197-15207.
201. Iida, T., Onodera, K., and Nakase, H. (2017) Role of autophagy in the pathogenesis of inflammatory bowel disease, *World journal of gastroenterology 23*, 1944-1953.
202. Giannogonas, P., Apostolou, A., Manousopoulou, A., Theocharis, S., Macari, S. A., Psarras, S., Garbis, S. D., Pothoulakis, C., and Karalis, K. P. (2016) Identification of a novel interaction between corticotropin releasing hormone (Crh) and macroautophagy, *Scientific reports 6*, 23342.
203. Kabi, A., Nickerson, K. P., Homer, C. R., and McDonald, C. (2012) Digesting the genetics of inflammatory bowel disease: insights from studies of autophagy risk genes, *Inflammatory bowel diseases 18*, 782-792.
204. Madsen, K., Cornish, A., Soper, P., McKaigney, C., Jijon, H., Yachimec, C., Doyle, J., Jewell, L., and De Simone, C. (2001) Probiotic bacteria enhance murine and human intestinal epithelial barrier function, *Gastroenterology 121*, 580-591.
205. Baksh, S., Widlund, H. R., Frazer-Abel, A. A., Du, J., Fosmire, S., Fisher, D. E., DeCaprio, J. A., Modiano, J. F., and Burakoff, S. J. (2002) NFATc2-mediated repression of cyclin-dependent kinase 4 expression, *Molecular cell 10*, 1071-1081.

206. Bjorkoy, G., Lamark, T., Pankiv, S., Overvatn, A., Brech, A., and Johansen, T. (2009) Monitoring autophagic degradation of p62/SQSTM1, *Methods in enzymology 452*, 181-197.

207. Mizushima, N., Yoshimori, T., and Levine, B. (2010) Methods in mammalian autophagy research, *Cell 140*, 313-326.

208. Petiot, A., Ogier-Denis, E., Blommaart, E. F., Meijer, A. J., and Codogno, P. (2000) Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells, *The Journal of biological chemistry 275*, 992-998.

209. Ito, S., Koshikawa, N., Mochizuki, S., and Takenaga, K. (2007) 3-Methyladenine suppresses cell migration and invasion of HT1080 fibrosarcoma cells through inhibiting phosphoinositide 3-kinases independently of autophagy inhibition, *International journal of oncology 31*, 261-268.

210. Ermann, J., Staton, T., Glickman, J. N., de Waal Malefyt, R., and Glimcher, L. H. (2014) Nod/Ripk2 signaling in dendritic cells activates IL-17A-secreting innate lymphoid cells and drives colitis in T-bet-/-.Rag2-/- (TRUC) mice, *Proceedings of the National Academy of Sciences of the United States of America 111*, E2559-2566.

211. Lupfer, C. R., Anand, P. K., Liu, Z., Stokes, K. L., Vogel, P., Lamkanfi, M., and Kanneganti, T. D. (2014) Reactive oxygen species regulate caspase-11 expression and activation of the non-canonical NLRP3 inflammasome during enteric pathogen infection, *PLoS pathogens 10*, e1004410.

212. Lupfer, C., Thomas, P. G., Anand, P. K., Vogel, P., Milasta, S., Martinez, J., Huang, G., Green, M., Kundu, M., Chi, H., Xavier, R. J., Green, D. R., Lamkanfi, M., Dinarello, C. A., Doherty, P. C., and Kanneganti, T. D. (2013) Receptor interacting protein kinase 2-mediated mitophagy regulates inflammasome activation during virus infection, *Nature immunology 14*, 480-488.

213. Canning, P., Ruan, Q., Schwerd, T., Hrdinka, M., Maki, J. L., Saleh, D., Suebsuwong, C., Ray, S., Brennan, P. E., Cuny, G. D., Uhlig, H. H., Gyrd-Hansen, M., Degterev, A., and Bullock, A. N. (2015) Inflammatory Signaling by NOD-RIPK2 Is Inhibited by Clinically Relevant Type II Kinase Inhibitors, *Chemistry & biology 22*, 1174-1184.

214. Haile, P. A., Votta, B. J., Marquis, R. W., Bury, M. J., Mehlmann, J. F., Singhaus, R., Jr., Charnley, A. K., Lakdawala, A. S., Convery, M. A., Lipshutz, D. B., Desai, B. M., Swift,

B., Capriotti, C. A., Berger, S. B., Mahajan, M. K., Reilly, M. A., Rivera, E. J., Sun, H. H., Nagilla, R., Beal, A. M., Finger, J. N., Cook, M. N., King, B. W., Ouellette, M. T., Totoritis, R. D., Pierdomenico, M., Negroni, A., Stronati, L., Cucchiara, S., Ziolkowski, B., Vossenkamper, A., MacDonald, T. T., Gough, P. J., Bertin, J., and Casillas, L. N. (2016) The Identification and Pharmacological Characterization of 6-(tert-Butylsulfonyl)-N-(5-fluoro-1H-indazol-3-yl)quinolin-4-amine (GSK583), a Highly Potent and Selective Inhibitor of RIP2 Kinase, *Journal of medicinal chemistry 59*, 4867-4880.

215. Tigno-Aranjuez, J. T., Benderitter, P., Rombouts, F., Deroose, F., Bai, X., Mattioli, B., Cominelli, F., Pizarro, T. T., Hoflack, J., and Abbott, D. W. (2014) In vivo inhibition of RIPK2 kinase alleviates inflammatory disease, *The Journal of biological chemistry 289*, 29651-29664.
216. Dorsch, M., Wang, A., Cheng, H., Lu, C., Bielecki, A., Charron, K., Clauser, K., Ren, H., Polakiewicz, R. D., Parsons, T., Li, P., Ocain, T., and Xu, Y. (2006) Identification of a regulatory autophosphorylation site in the serine-threonine kinase RIP2, *Cellular signalling 18*, 2223-2229.
217. Tigno-Aranjuez, J. T., Asara, J. M., and Abbott, D. W. (2010) Inhibition of RIP2's tyrosine kinase activity limits NOD2-driven cytokine responses, *Genes & development 24*, 2666-2677.
218. Liu, L., McKeehan, W. L., Wang, F., and Xie, R. (2012) MAP1S enhances autophagy to suppress tumorigenesis, *Autophagy 8*, 278-280.
219. Hooper, K. M., Barlow, P. G., Stevens, C., and Henderson, P. (2017) Inflammatory Bowel Disease Drugs: A Focus on Autophagy, *Journal of Crohn's & colitis 11*, 118-127.
220. Fujiwara, N., Usui, T., Ohama, T., and Sato, K. (2016) Regulation of Beclin 1 Protein Phosphorylation and Autophagy by Protein Phosphatase 2A (PP2A) and Death-associated Protein Kinase 3 (DAPK3), *The Journal of biological chemistry 291*, 10858-10866.